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The Possible Association Between Porphyrins and Cancer in Mice*

John J. Bittner, Ph.D., and Cecil James Watson, M.D., Ph.D.

With the Technical Assistance of Violet Hawkinson and Eleanore A. Anderson

(From the Department of Physiology, Division of Cancer Biology and Medicine, University of Minnesota Medical School, Minneapolis 14, Minnesota)

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Since a method was available (13) that could easily be adapted to a quantitative determination of porphyrins in the harderian glands of mice, it seemed advisable, in view of recent publications by others, to ascertain if there might be any relationship between porphyrin metabolism and the possible causes of the various types of cancer, especially mammary cancer, in mice.

Strong and Figge (18) and Figge and his group (12) have made estimates of the degree of red fluorescence of the porphyrins in the harderian glands of mice, by visual inspection and comparison under near-ultraviolet light. They found that there was a rapid increase in red fluorescence soon after the eyes were open, until a maximum was reached at approximately 100 days of age in female mice. After that age the decrease in the red fluorescence was dependent somewhat upon the strain studied. In mice of the low-cancer JK strain the red fluorescence completely disappeared in middle sexual maturity, and this absence persisted for the remainder of life, whereas those of the cancerous C3H stock showed a slower decline in the intensity with advancing age. The authors stated that, in general, mice of strains with a high incidence of mammary cancer showed the maximal red fluorescence of the harderian glands, although some mice of the cancerous A stock gave readings that were characteristic for mice with intermediate degrees of susceptibility. The authors interpreted their data as evidence for the hypothesis that there is either a direct or an indirect relationship between porphyrin metabolism and inherited susceptibility to mammary cancer. It was concluded that a relatively large production of porphyrins in the harderian glands may be inherited as a dominant.

Figge (11) has further postulated some relationship between excess porphyrins (or a unique metabolism) and the susceptibility to carcinogenic agents.

Following the study of reciprocal hybrids between the cancerous C3H and the low-cancer JK stock, Strong (17) reported the following:

(a) In all groups the degree of red fluorescence increased from the time the eyes opened until early sexual maturity was reached, and then declined gradually.

(b) In all groups the fluorescence intensity for the females was greater than for the males of the same strain.

(c) The fluorescence for mice of the F_1 generation was intermediate in intensity between the two ancestral stocks.

(d) The fluorescence intensity noted in animals of the F_1 generation was closer to that of the female ancestral stock than to that of the paternal strain.

Strong concluded that whether porphyrins have any effect in the etiology of cancer or not must be tested by the suitable administration of porphyrins to experimental animals.

MATERIAL AND METHOD

STOCKS OF MICE

The various strains of mice and their hybrids used in this study, with their approximate incidence of

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spontaneous mammary cancer, are given in Table I. Because of certain points to be discussed later we have indicated whether or not the virgin females of the respective groups have the milk agent, the inherited susceptibility, and the inherited hormonal influence, all of which are generally necessary for the development of spontaneous mammary cancer in virgin females (2, 3, 9, 15, 10). Breeding females of the respective groups would have the same inciting influence and, in addition, the increased hormonal stimulation resulting from the bearing of young.

The animals were fed Purina fox chow and had an unlimited supply of tap water.

The technic described by Figge and his associates (12) for removal of the harderian glands was followed.

TABLE I: INCITING INFLUENCE FOR MAMMARY CANCER IN THE VIRGIN FEMALES OF THE VARIOUS GROUPS USED, WITH THE INCIDENCE OF MAMMARY CANCER

No group is listed as noncancerous, although some have remained free from mammary cancer for several years.

Stock	Matings	Milk agent	Inherited susceptibility	Inherited hormonal influence	Approximate incidence in virgins, per cent	Approximate incidence in breeders, per cent
A	A♀ × A♂	+	+	—	3	90
Ax	Ax♀ × Ax♂	—	+	—	1	1
Z (C3H)	Z♀ × Z♂	+	+	+	63	90
Zb	Zb♀ × Zb♂	—	+	+	1	1
AZF_1	A♀ × Z♂	+	+	+	90	90
ZAF_1	Z♀ × A♂	+	+	+	75	90
$AxZbF_1$	Ax♀ × Zb♂	—	+	+	1	1
$ZbAxF_1$	Zb♀ × Ax♂	—	+	+	1	1
D (dba)	D♀ × D♂	+	+	+	50 *	85 *
B (C57 blk.)	B♀ × B♂	—	? †	—	1	1

* Data incomplete and based on small numbers represent mice from 3 sublines.

† Mammary cancer will develop in some animals with the milk agent, but the inherited susceptibility may vary with the subline.

PORPHYRIN DETERMINATION

The harderian glands [1] from a group of animals, varying in number in different experiments, were weighed wet and then minced and ground in an evaporating dish with a small amount of glacial acetic acid. The method used in extracting protoporphyrin from erythrocytes (13) was employed. Three parts of ethyl acetate were added and thoroughly ground together with the mixture, after which the residue was allowed to settle to the bottom of the dish and the supernatant fluid was decanted through a filter paper into a separatory funnel. The acetic-acid-ethyl acetate extraction was repeated several times, until no more fluorescence was observed.

The combined extracts were washed twice with distilled water and the washings examined carefully for porphyrin. If porphyrin was extracted by the glands the protoporphyrin was isolated in the usual way, the crystalline methyl ester melting at 227° C. (corrected). No evidence was found to indicate the presence of any porphyrin other than protoporphyrin.

[1] Direct inspection of the glands in ultraviolet light revealed that the red fluorescence was most intense at the surface but it was evident that some porphyrin was contained in the substance of the gland also.

The porphyrin was next removed from the ethyl acetate by several extractions with small amounts of 5 per cent HCl. The red fluorescence was quantitated in a Klett fluorophotometer against a 3 mgm. per cent fluorescein standard. The readings for a solution of crystalline protoporphyrin in 5 per cent HCl are given in Table II.

In the tables the readings are expressed either as the number of gamma of porphyrin per 100 mgm. of harderian gland (γ/100 mgm.) or gamma per entire gland (γ/ gland).

From the combined extracts of a large number of water because of excess acetic acid, it was re-extracted with ethyl acetate, which was then added to the original extract.

TABLE II: FLUORESCENCE INTENSITY OF PROTOPORPHYRIN IN 5 PER CENT HCl, READ AGAINST A 3 MGM. PER CENT FLUORESCEIN SOLUTION IN A KLETT FLUOROPHOTOMETER

Per 100 cc.	Klett reading
10	10
20	24
30	38
40	48
50	60
60	70
70	80
80	90
90	100
100	108

The calculation of protoporphyrin concentration in the harderian glands is as follows:

$$\gamma \text{ per 100 cc.} \times \frac{\text{cc. of final 5 per cent HCl solution}}{100} \times \frac{100}{\text{mgm. gland extraction}} = \gamma \text{ protoporphyrin per 100 mgm. of gland.}$$

TABLE III AVERAGE SIZE AND PORPHYRIN CONTENT OF HARDERIAN GLANDS FROM MICE OF THE Z (C3H) AND A STOCKS IN RELATION TO THE MILK AGENT

Stock		Data from Mice with Milk Agent				Data from Mice without Milk Agent			
		No.	Aver. wt.	γ/100 mgm.	γ/gland	No.	Aver. wt.	γ/100 mgm.	γ/gland
Z (C3H)	Virgin	32	.0182	90.6	16.5	24	.0194	83.5	16.2
A	Virgin	32	.0262	21.8	5.7	47	.0226	17.7	4.0
Average	Virgin	64	.0222	56.2	11.1	71	.0210	50.6	10.1
Z (C3H)	Breeder	21	.0259	169.8	44.0	30	.0248	215.5	53.4
A	Breeder	25	.0319	44.1	14.1	28	.0348	28.9	10.1
Average	Breeder	46	.0289	107.0	29.1	58	.0298	122.2	31.8
Z (C3H) and A	Virgin and Breeder	110	.0256	81.6	20.1	129	.0254	86.4	21.0

EXPERIMENTAL RESULTS

Mice of comparable ages of the A and Z (C3H) stocks, with and without the milk agent, are contrasted in Table III as to the porphyrin content of their harderian glands and the average weight of the glands in grams. Table IV gives similar data for breeding females of the cancerous A and Z stocks, 76 per cent of which had mammary cancer at the time they were used; and for breeding females of these stocks of the same ages, which were free of the milk agent and in which no mammary tumors have been observed during the past 3 years (Ax and Zb lines). Whereas the mice of the A stock with the milk agent showed slightly higher readings than did mice without the agent (Ax line), the opposite was the case for the two lines of the Z stock. The averages for the two groups (virgins and breeders) were approximately the same; mice without the milk agent had slightly higher readings, but the difference was probably not significant.

TABLE IV: DETERMINATIONS OF PORPHYRINS IN HARDERIAN GLANDS OF MICE OF THE A AND Z (C3H) STOCKS WITH AND WITHOUT THE MILK AGENT

Seventy-six per cent of the mice with the agent had mammary cancer.

Stock	No.	Cancerous	Aver. wt. of gland, gm.	γ/100 mgm.	γ/gland
A	20	14	.0388	42.0	16.3
Z (C3H)	22	18	.0251	169.0	42.4
Ax	19	0	.0282	37.7	10.6
Zb	31	0	.0239	221.2	52.9

Since these comparisons did not indicate any relationship between the porphyrin content of the harderian glands and the active milk agent, the data obtained for mice with and without the agent of the A and Z (C3H) stocks and their reciprocal F_1 hybrids have been combined in subsequent tabulations. The porphyrin determinations are presented in Table V according to age. The average weights of the glands in grams and the number of mice

TABLE V: PORPHYRIN DETERMINATIONS FOR MICE OF VARIOUS STOCKS ACCORDING TO AGE EXPRESSED IN GAMMA PER 100 MGM. OF TISSUE OR GAMMA PER GLAND

Stock		Total no.	4-6 wks.		2-7 mos.		8-13 mos.		14-18 mos.		18 + mos.	
			γ/100 mgm.	γ/gland	γ/100 mgm.	γ/gland	γ/100 mgm.	γ/gland	γ/100 mgm.	γ/gland	γ/100 mgm.	γ/gland
Z (C3H)	Vg. ♀	83	27.5	3.3	91.4	17.4	83.0	23.2	155.4	34.2	121.9	28.2
	Br. ♀	75	—	—	164.8	42.0	202.4	48.8	227.6	54.2	—	—
A	Vg. ♀	80	25.8	3.2	22.4	5.5	—	—	—	—	16.7	5.0
	Br. ♀	92	—	—	33.9	11.5	43.9	14.1	26.1	8.6	42.2	15.4
F_1 AxZ	Vg. ♀	74	—	—	—	—	40.5	13.9	76.9	24.5	65.9	19.5
	Br. ♀	70	—	—	97.0	27.5	136.9	42.0	121.6	40.4	165.9	54.1
D (dba)	Vg. ♀	58	5.5	0.9	25.1	5.4	51.8	14.5	—	—	177.0	56.1
	Br. ♀	14	—	—	33.4	5.8	120.8	30.2	105.4	24.0	—	—
B (C57)	Vg. ♀	35	—	—	2.2	0.5	1.3	0.4	—	—	1.4	0.4
	Br. ♀	12	—	—	—	—	5.1	1.9	6.3	2.8	6.2	2.0
F_1 ZxD	Vg. ♀	9	—	—	41.3	9.6	43.6	11.9	—	—	—	—
	Br. ♀	6	—	—	—	—	206.0	63.5	—	—	—	—
A	Spayed ♀	5	—	—	—	—	—	—	—	—	78.8	20.7
ZBC	Spayed ♀	6	—	—	—	—	—	—	—	—	100.2	34.7

killed during each age period are given in Table VI. From the tabulations it is obvious that while the data are not so extensive as might be desired some general conclusions may be drawn.

In virgin females of the Z stock there was a gradual increase in the amount of porphyrin that could be extracted from the harderian glands, from the time the animals were 4 weeks of age until they had attained the age of 18 months. One reading for mice 22 months of age was slightly lower than that for animals 18 months of age, but it was higher than any secured for mice of other ages. From 2 to 18 months of age the breeding females of the Z stock had higher readings than did the virgin females. Breeders of 2 to 7 months of age had heavier glands than did the virgin females, but after 8 months no difference in size was apparent.

There was a slight increase in the harderian gland porphyrin from virgin females of the A stock of 4 weeks to 2 months of age, but by 6 months the amount was approximately the same as in 4 week old animals.[2] Virgin females from 7 to 17 months of age were not available for the study, but those that were 18 to 20 months of age did not show any decrease. Breeding females of the A stock had approximately the same amount of porphyrin during their entire life span, or until the age of 20 months. The harderian glands of the breeders were larger than those of virgins of the A stock and, because of this fact, the porphyrin content of the glands in these groups showed greater differences when the readings were obtained for the entire glands than for 100 mgm. of tissue.

Reciprocal F_1 hybrids between the A and Z stocks, with and without the milk agent, were available as virgins only between the ages of 8 and 24 months. The harderian glands removed from the hybrids were of approximately the same size as in the A stock, and larger than those from mice of the Z strain. Because of the larger glands in the F_1 virgins than in the Z virgins, the differences between the porphyrin content of the entire gland were not so great as the readings for 100 mgm. of tissue. Breeding F_1 females showed higher porphyrin readings at comparable ages than the virgin F_1 females. Because of the larger size of the harderian glands in the F_1 breeders, the porphyrin content of the entire gland was approximately the same as in breeding Z females. However, the hybrid mice attained their high level at a later age than did the Z animals. Based on the porphyrin content for 100 mgm. of gland, the readings for the breeding F_1 females were intermediate between those for A and Z breeders.

At comparable ages the average weight of the harderian glands from virgin females of the D stock was greater, based on small numbers, than the weight of glands from breeding females. From 4 weeks to 7 months of age the porphyrin content of glands from the D stock compared with the readings secured for mice of the A stock. After 8 months of age the increase was more rapid in the breeding females, and one group of virgin females, killed at 22 months of age, had the highest reading for virgins of any strain.

Five determinations were obtained on F_1 hybrids between the Z and D stocks. In virgin females 6 to 12 months of age the averages were about the same

TABLE VI: NUMBER OF MICE OF THE VARIOUS GROUPS KILLED DURING VARIOUS AGE PERIODS, AND AVERAGE WEIGHT OF HARDERIAN GLANDS IN GRAMS

Stock		4-6 wks.		2-7 mos.		8-13 mos.		14-18 mos.		18 + mos.	
		No.	Aver.	No.	Aver.	No.	Aver.	No.	Aver.	No.	Aver.
Z (C3H)	Vg. ♀	16	.0119	38	.0190	3	.0280	6	.0220	20	.0231
	Br. ♀	—	—	24	.0255	43	.0241	8	.0238	—	—
A	Vg. ♀	19	.0125	21	.0246	—	—	—	—	40	.0301
	Br. ♀	—	—	21	.0339	46	.0320	17	.0331	8	.0364
F_1	Vg. ♀	—	—	—	—	5	.0342	21	.0319	48	.0296
	Br. ♀	—	—	5	.0283	24	.0307	21	.0332	20	.0326
D (dba)	Vg. ♀	6	.0161	26	.0215	23	.0280	—	—	3	.0317
	Br. ♀	—	—	4	.0175	7	.0250	3	.0227	—	—
B (C57)	Vg. ♀	—	—	12	.0224	6	.0302	—	—	17	.0283
	Br. ♀	—	—	—	—	6	.0371	2	.0446	4	.0321
F_1 (ZxD)	Vg. ♀	—	—	3	.0233	6	.0272	—	—	—	—
	Br. ♀	—	—	—	—	6	.0308	—	—	—	—
A	Spayed ♀	—	—	—	—	—	—	—	—	5	.0263
ZBC	Spayed ♀	—	—	—	—	—	—	—	—	6	.0348

[2] Four week old animals of the A and Z stocks gave approximately the same readings. The more rapid increase in mice of the Z stock is not apparent in the table because of the small number of mice killed at 6 weeks of age as contrasted with the number killed when they were 4 weeks of age.

as for virgin females of the D stock; in other words, lower than for the Z stock. The single reading for breeding females of the ZDF_1 hybrid generation compared well with the content of glands from breeding females of the Z stock of the same age. The figure for the whole glands was the highest obtained in any group.

While the average weight of the harderian glands from virgin females of the B (C57 black) low-cancer stock was no greater than in A virgins, breeding females of the B stock had the largest glands of any group. While there was a slight increase in the porphyrin content in glands from the breeding females, they had readings that were lower than those for virgin females of any other stock.

With the cooperation of Miss Fern Smith it was possible to obtain readings on 2 groups of ovariectomized mice (16): animals of the A stock that were 20 months of age, and ZBC ($ZbAxF_1 ♀ x Z♂$) females of 23 months. The porphyrin content of the harderian glands from the spayed A females was higher than for any other group, breeder or virgin, of the A stock. The reading for the castrated ZBC females was higher than for any group of AZF_1 virgins, and compared with virgin Z females of 18 months.[3]

DISCUSSION

It is evident that the data submitted above are not adequate to permit a complete analysis of porphyrin metabolism in the different strains of mice. However, from these observations, involving 178 determinations on over 600 mice, some general conclusions may be drawn on the possible role of the porphyrins in the etiology of cancer in mice, and especially of mammary cancer.

We were unable to find any correlation between the active milk agent for mammary cancer and the amount of porphyrin present in the harderian glands.[4] That is, different lines of the same inbred stock, one with and the other without the agent, gave approximately the same reading for mice of comparable ages. No evidence has been obtained that foster nursing of mice of cancerous stocks (2), with the elimination of the active milk agent, will influence the inherited susceptibility for the development of spontaneous mammary cancer (4-7). Also, this susceptibility may be transmitted by males as well as by females of the susceptible stock.

Based on the porphyrin content of 100 mgm. of gland, our findings would tend to confirm those of Figge and his associates (12), and of Strong (17), in that the porphyrin content in hybrids was intermediate between the parental strains. However, when readings for the entire gland were considered the hybrids might compare with the parental stock with the higher amount, except that the maximal level in the hybrids was attained at later ages. This variation in the readings between 100 mgm. of tissue and the entire gland was due primarily to the difference in the average weights of the harderian glands in the mice of the various groups. Although other workers (12) have stated that the degree of red fluorescence was lost at different ages by mice of different stocks, we could find no apparent reduction in the amount of porphyrin that could be extracted from the harderian glands with increasing age. The difference in the method of determination may account for this, since Figge (11) stated: "The extraction of some of the non-red-fluorescence showed that porphyrins were present, but the concentration was not high enough to be detected by fluorescence in near ultraviolet light."

No evidence has been advanced to indicate that mice of the Z or C3H strain are more "susceptible" to the development of mammary cancer than those of the A stock. The breeding females of the two strains employed in the present study have approximately the same incidence (5, 6, 10). While the virgin females of the C3H stock have a much higher incidence than virgin females of the A stock, this difference results primarily from genic control of the hormonal mechanism (9, 15): a condition called the inherited hormonal influence. The genic make-up of the inherited susceptibility is not the same as the inherited hormonal influence (10).

When reciprocal hybrids were produced by mating mice of the cancerous A and Z stocks, the results showed that either the two strains had the same susceptibility for mammary cancer, or, if different genes were transmitted, any combination of these genes would produce susceptibility in the F_2 generations (10). Unless we are able to detect a difference in the inherited susceptibility to mammary cancer in these two strains, for which there is no evidence at present, it would follow that the mice of these two cancerous stocks should have approximately the same porphyrin readings if there be any association between their porphyrins and their inherited susceptibility to spontaneous mammary cancer. This was not the case, and furthermore, because mice of the dilute brown stock, another cancerous strain, behaved quite differently from either of the other two cancerous stocks with respect to the porphyrin content of the

[3] Exophthalmos was noted in the spayed ZBC females, but not in the A animals, and the ZBC mice bled profusely when the harderian glands were removed.

[4] An extract of harderian glands from mice with the milk agent, diluted 1:100, has been shown to have the active agent (unpublished data).

harderian glands, we were unable to find any correlation between the inherited susceptibility to mammary cancer and the porphyrins such as has been postulated by others (11, 17).

This difference of opinion may be due to interpretation of the data in the two experiments. Figge and his associates (11) stated that "one apparent exception to the rule that mice with high susceptibility to spontaneous cancer show maximal red fluorescence of the harderian gland was seen in mice of the A stock." Of the 13 inbred strains of mice that they used in their study only 2, the C3H and the A, showed "high susceptibility" to spontaneous mammary cancer. For this reason their conclusion that a correlation exists does not appear to be warranted.

In every group employed in the present study, where it was possible to make comparisons, the breeding females showed higher readings than did the virgin females of the same stock. From this it would appear that the hormones may influence, in some manner, porphyrin metabolism.

It is of interest to note that the highest readings were observed in mice of stocks that were capable of developing a high incidence of mammary cancer in the virgins. Previous observation (9, 15, 8, 10) have shown that only the virgin females of strains with the inherited hormonal influence have a high incidence of mammary cancer. However, if there is any association between the porphyrins of the harderian glands and the inherited hormonal influence, some physiological effects of which have not yet been determined, the present data would imply that mice of the various stocks with the influence do not respond alike, for in the various groups the maximal readings might be attained at different ages. These differences may be effects of hormonal activity such as are reflected by variation in the average cancer age. On the other hand, if there is any relationship between porphyrin metabolism and the hormones, the high values observed in the 2 groups of spayed females would suggest that at least one of the principal hormones may be involved, the physiological effects of which have not been previously associated with those of the inherited hormonal influence.

These conclusions were made possible only because the readings for virgin females were compared with those of breeding females of the same strain. The other workers (12, 11, 17) have not differentiated between the two groups, although they found that females had higher degrees of fluorescence than males.

While Figge (11) has also assumed that the porphyrins may play some role in reflecting the susceptibility of tissues to various carcinogenic agents, the studies of Andervont (1) and Heston (15), correlated with our findings, would not support such a theory for the induction of lung tumors. As for spontaneous lung cancer, it was common in old mice of the A stock, especially those of the fostered line, in which the readings were low to intermediate; rare in mice of the Z stock with high readings; and common in the F_1 hybrids between the A and Z stocks without the milk agent and with high readings.

Thus it is possible that the primary relationship between the porphyrins and cancer may be an association between the porphyrins and the hormones.

SUMMARY

The extraction of porphyrins from the harderian glands of virgin and breeding females of various inbred stocks of mice and F_1 hybrids would suggest that:

There is no decrease in the porphyrin content of the harderian glands with increasing age.

There is no relationship between the presence of the active milk agent and the porphyrins.

There is no simple correlation between "inherited susceptibility" to mammary and lung cancer and the porphyrins.

In every stock the breeding females showed higher porphyrin concentrations in the harderian glands than did the virgin females. Spayed females may show higher reading than virgin females of the same stock.

The maximal concentrations in the various groups were attained at different ages.

The apparent relationship between the porphyrins and mammary cancer, as described by others, may be due to a correlation of unknown nature between the porphyrins and the hormones. In virgin females this association may involve the "inherited hormonal influence."

REFERENCES

1. Andervont, H. B. Pulmonary Tumors in Mice. II. The Influence of Heredity upon Lung Tumors Induced by the Subcutaneous Injection of a Lard-Dibenzanthracene Solution. Pub. Health Rep., **52**:304-315. 1937.
2. Bittner, J. J. Some Possible Effects of Nursing on the Mammary Gland Tumor Incidence in Mice. Science, **84**:162. 1936.
3. Bittner, J. J. "Influences" of Breast-Cancer Development in Mice. Pub. Health Rep., **54**:1590-1597. 1939.
4. Bittner, J. J. Breast Cancer and the Pedigree Relationship of Fostered A Stock Mice. Pub. Health Rep., **54**:1642-1650. 1939.
5. Bittner, J. J. Foster Nursing and Genetic Susceptibility for Tumors of the Breast in Mice. Cancer Research, **1**: 793-794. 1941.
6. Bittner, J. J. Mammary Cancers in Fostered and Unfostered C3H Breeding Females and Their Hybrids. Cancer Research, **3**:441-447. 1943.
7. Bittner, J. J. The Genetics and Linkage Relationship of the Inherited Susceptibility to Mammary Cancer in Mice. Cancer Research, **4**:779-784. 1944.

8. Bittner, J. J. Inciting Influences in the Etiology of Mammary Cancer in Mice. A. A. A. S., Research Conference on Cancer, F. R. Moulton, Ed. 1945, pp. 63-95.
9. Bittner, J. J., Huseby, R. A., Visscher, M. B., Ball, Z. B., and Smith, F. W. Mammary Cancer and Mammary Structure in Inbred Stocks of Mice and Their Hybrids. Science, **99**:83-85. 1944.
10. Bittner, J. J., and Huseby, R. A. Relationship of the Inherited Susceptibility and the Inherited Hormonal Influence in the Development of Mammary Cancer in Mice. Cancer Research, **6**:235-239. 1946.
11. Figge, F. H. J. Fluorescence Studies on Cancer. I. Porphyrin Metabolism, Harderian Gland Fluorescence, and Susceptibility to Carcinogenic Agents. Cancer Research, **4**:465-471. 1944.
12. Figge, F. H. J., Strong, L. C., Strong, L. C., Jr., and Shanbrom, A. Fluorescent Porphyrins in Harderian Glands and Susceptibility to Spontaneous Mammary Carcinoma in Mice. Cancer Research, **2**:335-342. 1942.
13. Grinstein, M., and Watson, C. J. Studies of Protoporphyrin. III. Photoelectric and Fluorophotometric Methods for the Quantitative Determination of the Protoporphyrin in Blood. J. Biol. Chem., **147**:675-684. 1943.
14. Heston, W. E. Genetic Analysis of Susceptibility to Induced Pulmonary Tumors in Mice. J. Nat. Cancer Inst., **3**:69-78. 1942.
15. Heston, W. E., and Andervont, H. B. Importance of Genetic Influence on the Occurrence of Mammary Tumors in Virgin Female Mice. J. Nat. Cancer Inst., **4**:403-407. 1944.
16. Smith, F. W. Castration Effects of the Inherited Hormonal Influence. Science, **101**:279-281. 1945.
17. Strong, L. C. Material Inheritance of Protoporphyrins on the Harderian Glands in Mice. Proc. Soc. Exper. Biol. & Med., **57**:78-79. 1944.
18. Strong, L. C., and Figge, F. H. J. Fluorescence of Harderian Glands in Mice of Cancer-Susceptible and Cancer-Resistant Strains. Science, **94**:331. 1941.

The Effects of Suramin (Germanin), Azo Dyes, and Vasodilators on Mice with Transplanted Lymphosarcomas*

W. Lane Williams, Ph.D.**

(Departments of Anatomy, Yale University School of Medicine, New Haven 11, Connecticut and Louisiana State University School of Medicine, New Orleans 13, Louisiana)

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INTRODUCTION

The neoplastic tissue used for transplantation originated as a mediastinal tumor in an estrogen-treated C3H mouse (17, 19, 20). This tumor, of thymic origin, is best classified on the basis of its morphology and growth characteristics as a lymphosarcoma. The same or similar types of lymphoid tumors developed in 14.4 per cent of 747 C3H mice treated with estrogenic sterols (19). The particular tumor used (6C3HED) has been transplanted through over 25 generations and has not metastasized, although its growth is rapid. The technic included subcutaneous and intraperitoneal grafting with a trocar and the injection at similar sites of tumor tissue suspended in normal saline (19, 55).

The subcutaneous transplant grows as a large mass, which is localized except for involvement of immediately adjacent lymph nodes; intrathoracic and intra-abdominal metastasis has not been observed. The intraperitoneal transplants have previously been described in detail (55).

In the original hosts these estrogen-induced lymphoid tumors of the mediastinum invaded adjacent structures (lymph nodes, heart, great vessels, thoracic wall, etc.) and in a considerable number of mice there were metastases to the liver, lungs, kidneys, and other viscera (19). For all these reasons, and because of the ready transplantability of the tumor, it seemed possible that local and systemic conditions might be produced in the recipient hosts that would influence the growth pattern of the transplant to the extent that widespread metastasis would occur. Therefore the original purpose of the study was to determine if treatment of the hosts with anticoagulants and vasodilators would cause metastasis of the transplanted tumor beyond adjacent lymph nodes. Suramin,[1] the sodium salt of symmetric bis (meta-amino-benzoyl-meta-amino-para-methylbenzoyl-l-naphthyl-amino-4,6,8-trisulfonic-acid) carbamide, and the dye, chlorazol fast pink, were used as anticoagulants (21, 28, 49, 50, 52, 57); histamine [2] and depropanex,[3] as vasodilators.

As is described below in the preliminary series these injected materials did not induce metastasis; but one of them, suramin, seemed obviously to inhibit the growth of the tumor transplants. To investigate further the possible retardation of tumor growth by suramin the study about to be described (see MATERIALS and METHODS) was initiated.

PRELIMINARY SERIES OF MICE

Twenty-eight C3H mice (100 to 120 days of age) received subcutaneous transplants of the lymphosarcomas. Beginning on the day of transplantation these mice received injections of one of the several substances daily for 10 days in amounts of 1 mgm. (in 0.1 cc. of distilled water) as follows: suramin, 10 animals; histamine, 4 animals; chlorazol fast pink, 6 animals. Similarly 4 animals received 0.1 cc. of depropanex daily, and 4 received 1 mgm. of histamine suspended in beeswax and mineral oil (histamine 100 mgm.; mineral oil 14 cc.; beeswax 6 cc.). Three of the suramin-treated animals died within a week. The remaining 25 were killed 6 days after the cessation of injections (16 days subsequent to transplantation) and autopsied carefully. No evidence of metastasis other than enlargement of adjacent lymph nodes was observed. The tumors found in the suramin group seemed about one-half

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[1] This drug (suramin) has been known under several proprietary names. The preferable designation in the United States of America and in the United Kingdom is *suramin* or *suramin sodium*. The particular product used in this study was "Naphuride" (Winthrop).

[2] Histamine dihydrochloride (Eastman).

[3] Depropanex = deproteinated pancreatic extract, generously supplied by Sharpe and Dohme.

the size of those in the mice treated with dyes and vasodilators, but they were neither measured nor weighed. The "obvious inhibition" in the growth of transplants in suramin-treated animals, mentioned above, is based entirely upon visual inspection; therefore this preliminary series of animals will not be further discussed. It is included here more or less as an introduction.

MATERIALS AND METHODS

Sixty-four C3H mice (90 to 100 days of age) received subcutaneous transplants of the lymphosarcoma. In addition to the substances mentioned above some of the animals were also injected with the azo dyes, trypan blue and trypan red (1 mgm. in 0.1 cc. of distilled water). Fresh histamine and suramin solutions were used. The depropanex solutions were kept at 6° C. Injections were begun on the seventh day after transplantation, when all the animals had palpable tumors. The injections were made in the dorsal lumbar region contralateral to the transplantation site and continued for 7 to 14 days (Tables I and II).

A group of 20 animals of approximately the same size and age as those above received transplants of the same tumor simultaneously but were given no treatment (Tables I and II). In addition to these animals over 500 C3H mice have received transplants of this tumor, which in all instances has grown rapidly.

An additional group of 10 C3H mice received a subcutaneous injection of 0.1 cc. of a saline suspension of the tumor (1 gm. of ground tumor in 200 cc. of normal saline), and injections of 1 mgm. of suramin (100 mgm. in 5 cc. of distilled water) were initiated simultaneously and continued on alternate days for 10 days (Table I).

On the 21st day subsequent to transplantation the mice were killed with illuminating gas and complete autopsies were performed. The transplants were carefully dissected from the skin and muscle and the tumors and animals weighed. The tissues and organs were fixed in a formol-alcohol-acetic acid mixture (57). Sections were prepared by the paraffin method and stained with hematoxylin and eosin.

TABLE I

C3H mice received subcutaneous transplants of a lymphosarcoma and were killed 21 days later. During the last 7 to 16 days of this period the animals were treated as follows:

No. of animals	Treatment	Period of treatment, days	Effects of treatment on growth of transplants	Effects of treatment on other tissues
20	Untreated controls	Controls	Controls	Controls
17	1 mgm. of suramin daily (subcutaneously; 0.1 cc. aqueous sol., 1 gm./100 cc.)	7-11	Wt. 67.5% less than controls	Damage to lymph nodes, spleen, and kidneys
4	Trypan blue (Color Index no. 477) 1 mgm. as above	14	None	Usual dye storage
3	Trypan red (Color Index no. 438) 1 mgm. as above	10	"	Same
7	Chlorazol fast pink (Color Index no. 353) 1 mgm. as above	12-16	"	"
6	Histamine dihydrochloride, 1 mgm. as above	10	"	None
4	Depropanex subcutaneously, 0.1 cc. saline solution containing 10 depressor units per cc. (assayed as to effect upon arterial blood pressure of dogs)	14	"	"
	C3H mice received subcutaneous transplants of same tumor (as above) suspended in saline (1 gm. of ground tumor tissue in 200 cc. of normal saline); treated as described below, and killed 21 days after transplantation.			
10	1 mgm. of suramin as above on alternate days during first 10 days after transplantation	5 injections over a period of 5 days	Within control range in wt. at 21 days	None at 21 days

FINDINGS

1. Mice treated with *azo dyes, histamine,* and *depropanex:* With the exception of suramin the injected materials had little effect upon the general health of the animals. The vital dyes were stored in the usual fashion by macrophages and were also present in certain portions of the nephron. These animals showed the expected prolongation of coagulation and bleeding times (49, 50, 57). The dyes also demonstrated the presence of many macrophages in

the transplants (57). However, these phagocytes contained only the stored dye and no cellular remnants. The animals treated with histamine and depropanex showed no obvious deviations from normal. These, as well as the untreated controls, had large tumors, which had invaded the adjacent skin and muscle. There was no gross evidence or hemorrhage, ulceration, or infection. It is usual for these large transplants to show small focal areas of bacterial infection, but the organisms do not seem to interfere with their growth. Adjacent lymph nodes (axillary and/or inguinal) were greatly enlarged. In both gross and microscopic characteristics the tumors of animals treated with dyes, histamine, and depropanex were identical with those of untreated animals. The average weights of the tumors of the dye-treated (13 gm.), histamine-treated (9.8 gm.), and depropanex-treated animals (10.0 gm.) did not vary significantly (Fig. 1) from those of the controls (10.8 gm.).

2. The *suramin*-treated mice were relatively inactive during the last week of the experiment; three died during the first week of treatment. There was no appreciable loss in body weight (Figs. 1 and 3, and Table II), and the usual fat stores were not depleted. Although bleeding and clotting times were prolonged there were no gross hemorrhagic lesions. The transplants were much smaller than those observed in the controls and in the treated animals described above. In the suramin-treated animals they ranged from 1 to 7 gm. in weight (av. 3.5 gm.); in the untreated animals, from 7.3 to 16 gm. (av. 10.8 gm.). On the basis of these weights the tumors of the suramin-treated animals were 67.5 per cent smaller than those of the controls. When expressed in terms of the weight of tumor per gram of body weight the transplants of the treated animals were 60.9 per cent smaller. The weights of the tumors are shown in Table II, and Figs. 1, 2, and 3. Such tumors were relatively small localized masses, usually attached firmly to the dermis and subcutaneous connective tissues but in most cases separable from the underlying muscle with ease. The adjacent lymph nodes were either of normal size or slightly enlarged. The tumors were very edematous, but there was no evidence of hemorrhage in the fresh specimens or in the histological preparations. The latter showed clearly the presence of edema, necrotic tumor cells, free remnants of dead cells, and many macrophages containing such remnants (Figs. 5 to 9). Necrosis was limited to the tumor lymphocytes; all other elements appeared to be the same as in untreated animals. That the macrophages possessed their usual phagocytic abilities was

TABLE II

Controls				Suramin-treated				
Mouse no.	Tumor wt., gm.	Animal wt.,* gm.	Ratio Tumor wt. / Body wt.* gm.	Mouse no.	Tumor wt., gm.	Animal wt.,* gm.	Ratio Tumor wt. / Body wt.,* gm.	Period of treatment, days
6	16.0	22.0	0.73	1a	7.0	19.1	0.36	10
18	14.2	29.8	0.48	18	6.0	20.0	0.30	9
7	13.7	26.3	0.52	4	5.0	21.5	0.23	7
8	13.4	20.2	0.66	17a	5.0	26.0	0.15	9
5	13.5	22.5	0.60	2	4.5	21.1	0.21	10
17	11.7	28.3	0.41	16a	4.0	25.0	0.16	9
1	11.0	24.0	0.46	13	4.0	26.0	0.15	9
9	11.0	26.2	0.41	5	4.0	22.0	0.18	7
16	10.5	29.5	0.36	15	3.8	26.2	0.14	9
20	10.4	22.6	0.46	19	3.6	18.6	0.19	9
10	10.2	24.4	0.42	3	3.0	18.2	0.16	11
4	10.0	21.0	0.48	16	2.7	23.3	0.11	9
19	10.0	26.0	0.38	17	2.7	18.3	0.15	9
12	10.0	20.0	0.50	10	1.6	17.6	0.09	7
13	10.0	19.0	0.53	14	1.4	25.6	0.05	9
11	9.0	23.2	0.38	11	1.0	20.0	0.05	9
2	9.0	23.0	0.39	12	1.0	25.0	0.04	9
21	8.8	23.2	0.38					
3	8.0	20.5	0.39					
15	7.3	32.7	0.22					
Mean	10.8	24.2	0.41		3.5 (67.5% less than controls)	21.9 (9.5% less than controls)	0.16 (60.9% less than controls)	

* Body weight of mouse after removal of tumor.

well demonstrated (Fig. 7). Many of the tumor lymphocytes stained poorly and their nuclei were

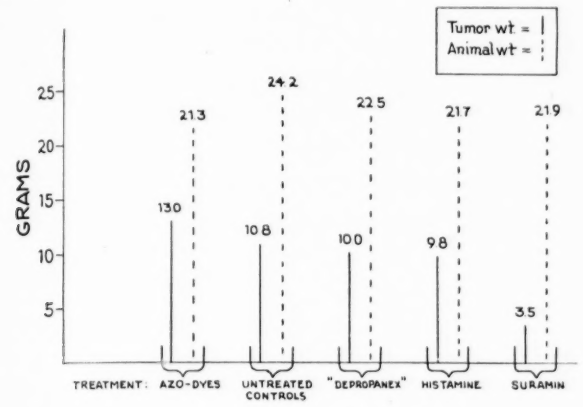


FIG. 1.—Average weights of lymphosarcomas and of mice at 21 days after transplantation. Treatment is shown in Table I.

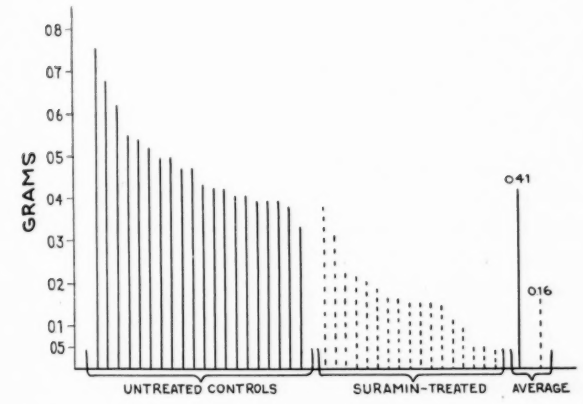


FIG. 2.—Weights of lymphosarcomas at 21 days subsequent to transplantation. The 17 treated mice shown in the figure received 1 mgm. of suramin daily during the terminal 7 to 11 days of the 21 day period.

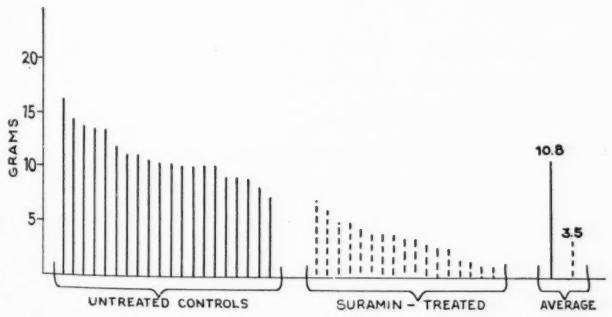


FIG. 3.—Ratio of tumor (lymphosarcoma) weight to body weight $\frac{\text{(tumor weight, gm.)}}{\text{(body weight, gm.)}}$ at 21 days after transplantation. The 17 treated mice shown received 1 mgm. of suramin daily during the terminal 7 to 11 days of the 21 day period.

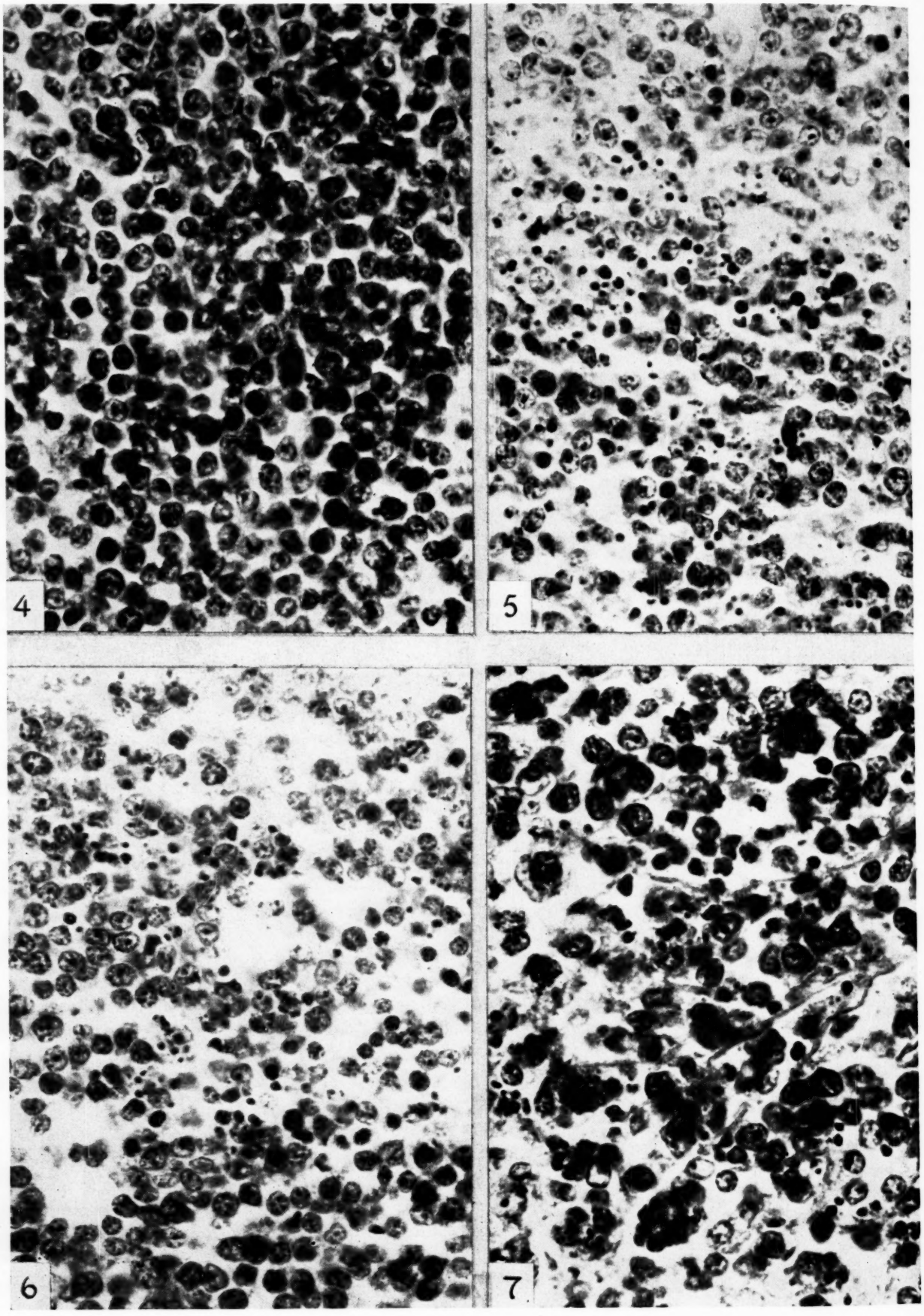
more vesicular and less basophilic than usual (Figs. 5 and 6). These cells seemed delicate and fragile. All the transplants contained neoplastic lymphocytes, which did not differ morphologically from those of the controls (Figs. 5 to 7). The number of mitoses was greatly reduced, but usually at least one was present in a single high-power field (Fig. 5 and 6). This was in striking contrast to the large number present in the tumors from animals that had not been treated with suramin (Fig. 4).

It should be stated here that tissue from 2 tumors (each weighing 5 gm.) from suramin-treated animals was successfully transplanted to 10 C3H mice.

The *lymph nodes* and *spleens* also showed pronounced alterations in morphology. The cortices of the former revealed an extreme reduction in the number of lymphocytes and the usual nodular arrangement of these cells was lacking (Figs. 10 and 11); the only true lymphoid cells present were a few large lymphocytes. The other cellular elements were reticular cells, macrophages, fibroblasts, and plasma cells (Figs. 12 and 13). The vascular and sinusoidal systems of the nodes seemed unchanged except for the presence of some necrotic cells in the latter. The most striking alteration was the previously mentioned absence of true lymphoid nodules or areas in the cortex. Upon first impression this change seemed to consist entirely of a depletion of the usual lymphoid cells, but closer examination revealed the presence of a few necrotic cells with pyknotic nuclei as well as some trace of cellular remnants. However, the limited number of clearly necrotic cells was by no means in proportion to the apparent depletion of or decrease in lymphocytes. A few mitoses were present in the reticular cells. The changes that are described here were observed in mesenteric, peri-aortic, mediastinal, inguinal, and axillary nodes.

The damage to lymphoid elements was less pronounced in the spleen than in the lymph nodes. However, the usual diffuse and nodular collections of lymphocytes were reduced in number and size. In one spleen (that from an animal with one of the smallest tumors) there was widespread necrosis of lymphoid tissue (Fig. 14). In general the sinusoidal system of the spleens was extremely congested (Fig. 15).

The *kidneys* followed the tumors and lymphoid tissues in order of frequency of morphological change resulting from the suramin treatment. They were slightly enlarged, brown in color, and edematous, there was swelling and necrosis of the epithelium of the convoluted tubules, and periglomerular and peritubular collections of leukocytes were present. The epithelium of the collecting tubules was enlarged and there was interstitial edema. The total amount of renal damage varied from focal lesions in one, to generalized involvement of both kidneys.



FIGS. 4-7

The *livers* of 4 suramin-treated animals were lighter than usual in color and in 2 cases there were small focal abscesses. With the exception of these the hepatic changes consisted of a slight perilobular leukocytosis and a minor degree of diffuse, irregular necrosis of parenchymal cells.

The *adrenals* showed generalized vascular congestion, particularly in the cortex. The boundaries of the zona fasciculata were less definite and the zona reticularis was slightly wider than usual.

There were no significant changes observed in alimentary, nervous, respiratory, and reproductive systems.

3. The absence of effects of *early suramin treatment on the success of transplantation:* The 10 animals that had received 1 mgm. of suramin on alternate days for the first 10 days subsequent to transplantation showed no effects from the treatment; their tumors were well within the control range (5 to 7 gm.) for 21 day old transplants derived from subcutaneously injected tumor cells suspended in saline (55). The dose of suramin used for these animals seemed too small to inhibit the growth of the transplant, and nontoxic in general as judged by the lack of significant effect on the lymphoid tissue and the kidney.

DISCUSSION

The findings demonstrate that suramin is another of the agents that elicit degenerative changes in lymphoid tissue, since it caused necrosis of some of the lymphocytes of the lymph nodes and spleen (Figs. 10 to 15). Similarly the compound damaged the lymphoid cells of a transplanted lymphosarcoma enough to inhibit clearly its growth (Figs. 4 to 9). But the effect was only temporary, since even the smallest tumors contained cytologically normal and actively dividing neoplastic lymphocytes; furthermore, the transplantability of tumors from suramin-treated mice showed the viability of some of their cells. The death of 3 animals and the damage to the kidneys in the others indicate that the amount of suramin injected was toxic, and close to the lethal dose. Thus suramin was deleterious to both normal and malignant lymphoid cells, but the latter were more susceptible to the compound.

Included among the agents that cause atrophic changes in lymphoid tissue are x-rays, radium, colchicine, and benzene. The effects of x-rays, benzene and several related substances, sulfa compounds, arsenite, organic arsenicals, and dietary restrictions on transplantable mouse leukemia have been described in considerable detail (13).

Morphologically the effects of suramin on normal lymphoid tissue resembled those of roentgen injury (9, 23, 44). The cytotoxic effect on the tumor was similar to that of colchicine on lymphoid tumors (36) except that there was no evidence of the peculiar mitotic alterations produced by that alkaloid. The karyoklastic action of colchicine is not limited to lymphocytes, but is effective systemically and locally (2, 35-37, 56, 58), though the lymphocyte seems to be most susceptible to it as well as to suramin (35, 36). These two substances are similar also in that the cytotoxic action of both is more selective for the neoplastic than the normal lymphocyte (36). *In vitro* studies indicate that certain of the tetramethyl-phenylenediamine compounds have fairly differential cytotoxic effects upon malignant lymphoid cells (4). The present study does not allow any conclusions as to the regenerative powers of damaged lymphoid tissue of the lymph nodes and spleen. A smaller dose of suramin had no effect upon transplantation or tumor growth (Table I) and produced no apparent changes in the organs.

Inanition is known to cause some atrophy of lymphoid tissues and to have some effect upon lymphogenous neoplasia (8, 27, 29). The short term of the present experiment and the failure of the treated mice to show significant loss in weight or depletion in fat stores does not indicate that inadequate general nutrition was the primary cause of the damage to the lymphoid tissues (Figs. 1, 2, and 3; Table II). All the animals were young and still growing, so it is difficult to draw any significant conclusions on the basis of absolute body weight. The average weight of the suramin-treated group was 9.5 per cent less than that of controls (Table II and Fig. 1). As was stated previously the animals treated with azo dyes and vasodilators seemed normal during life and at autopsy. The average weights of the dye-treated and histamine-treated mice were slightly less than

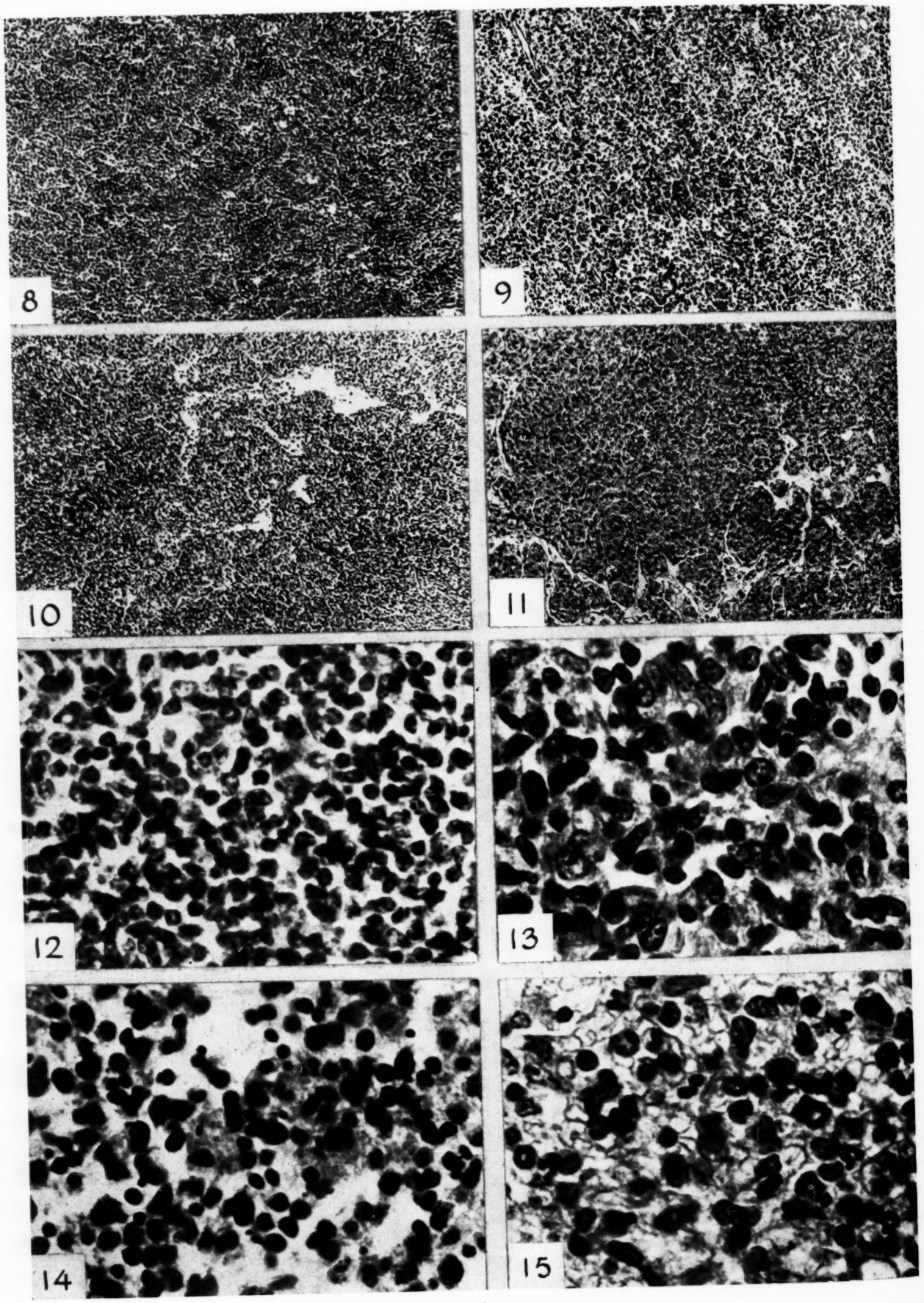
DESCRIPTION OF FIGURES 4 TO 7

(All mag. × 550)

Lymphosarcoma at 21 days after transplantation

FIG. 4 shows the dense arrangement of cells in tumors from untreated mice as well as numerous mitoses and well-staining hyperchromatic cells.

FIGS. 5 to 7 show transplanted lymphosarcoma tissue from 3 suramin-treated mice; observe necrotic cells, cellular debris, edema, macrophages (containing cellular remnants), infrequent mitoses, and apparently healthy tumor cells. In addition, many of the tumor cells have vesicular, poorly stained nuclei.



FIGS. 8-15

that of the suramin group (Fig. 1). There was no apparent relationship between body weight and tumor weight in control or treated animals. The largest animal (32.7 gm.) had the smallest tumor (7.3 gm.) among the group that was not treated with suramin. One of the 2 smallest tumors of the suramin-treated series was present in one of the larger animals (25 gm.) of this group. However, the general poor health of the suramin-treated mice (particularly the renal damage) must be considered as most probably related to the general processes or state that favored inhibition of tumor growth.

The x-ray, the carcinogenic hydrocarbons, and the estrogenic sterols have interesting and almost paradoxical effects upon lymphoid tissues. Normal and neoplastic lymphoid tissues are very susceptible to damage by x-rays (9, 44). On the contrary, a quantitatively small dose of x-rays seems to produce a slight amount of lymphoid destruction, which is quickly followed by stimulation (44). Roentgen-ray treatment alone, or in combination with the usual carcinogenic hydrocarbons, increases the incidence of lymphoid neoplasia (lymphomatoses and/or leukemia) in several strains of mice (3, 11, 12, 15, 16, 32, 34, 38), and the percutaneous application of carcinogens to several strains of mice causes a pronounced precocity in the occurrence of leukemia (30, 31, 41, 43, 48). Many of the experimental lymphoid neoplasms seem to originate in or from the usual lymphoid tissues (19, 29, 39). Of interest in this respect is the damage caused by another of the well known carcinogens (dibenzanthracene) to the lymphoid tissues of mice (25, 47). The estrogenic sterols definitely increase the incidence of lymphoid neoplasia in several strains of mice (17-20, 33). Transplants of such tumors do not depend upon estrogens in order to take and grow rapidly (19, 20, 55). Estrogens seem to cause atrophy of thymic and possibly other lymphoid tissues in the rat (51). In dogs, estrogens in large doses cause damage to both myeloid and lymphoid tissues (5, 54). The androgenic sterols decrease the incidence of lymphomatoses in estrogen-treated mice (19, 45). Adrenal cortical extracts and adrenocorticotrophic hormone deplete the lymph nodes of lymphocytes and inhibit lymphopoiesis in mice (6, 7, 42). The former material has some inhibitory effect upon transplanted lymphosarcoma in mice (24), and the latter upon leukemia in rats (46). A common basis for the various effects of x-ray, carcinogenic hydrocarbons, and estrogenic and other hormones on normal lymphoid tissues and their relationship to lymphoid neoplasia has not been found.

A subsequent study in rats has shown that lymphoid damage is probably the first manifestation of toxic doses of suramin. The animals show widespread terminal hemorrhagic lesions and severe anemia, but the lymphoid changes (depletion and necrosis of lymphocytes) precede these more general effects by 3 to 5 days (1). Other workers have described certain of the toxic effects of germanin on experimental animals, including rodents (26) but have not mentioned any considerable damage to the lymphoid tissues. Renal and adrenal cortical lesions were frequent (53).

In conclusion it should be stated that histamine, the three azo dyes, and depropanex were without obvious deleterious effects for mice in the doses used. Likewise these substances did not affect the growth of transplants. The materials used for injection in this study have interesting, and in some cases similar, pharmacological properties and effects. The trypan dyes (52), as well as suramin, exert trypanosomicidal effects (10, 21, 40). It seems worthy of mention that arsenicals, which are effective therapeutically in protozoan diseases also, have been reported to inhibit somewhat the clinical course of leukemia, including the lymphogenous type (14). The azo dyes, particularly chlorazol fast pink, are very effective anticoagulants in several species, including mice and rats (57), and the same is true for suramin (21, 52).

The attempt to produce definite metastasis of transplanted tumors by the use of vasodilators and anticoagulants was entirely unsuccessful.

Whether or not the apparent sensitivity of the neoplastic lymphocytes would allow the effective use of suramin in amounts that are not generally toxic cannot be stated. In general the present study seemed to indicate that it would not (29); the almost in-

DESCRIPTION OF FIGURES 8 TO 15

FIGS. 8 and 9.—The lymphosarcoma in an untreated mouse (Fig. 8) and in a suramin-treated mouse (Fig. 9). Mag. × 100.

FIGS. 10 and 11.—Cortical areas of mesenteric lymph nodes from a control mouse (Fig. 10) and a suramin-treated mouse (Fig. 11). Mag. × 100.

FIGS. 12 and 13.—Cortical areas in Figs. 10 and 11 at increased magnification (mag. × 550). In Fig. 12 (control mouse) the usual small and medium-sized lymphocytes are the predominant cells. Note in Fig. 13 the almost complete absence of these elements in the lymph node from a suramin-treated mouse. A few necrotic cells, as well as cells in mitosis, appear in the latter figure.

FIGS. 14 and 15.—Spleens from 2 suramin-treated mice. Observe necrotic cells, congestion, and scarcity of lymphocytes. Mag. × 550.

variable renal damage is an obvious limiting factor. It should also be clearly emphasized that damage to the kidneys and normal lymphoid organs was present even in mice that had received suramin in amounts only sufficient to *inhibit* the growth of their transplants.

SUMMARY AND CONCLUSIONS

1. The azo dyes had no effect upon the growth or morphology of transplanted lymphosarcomas, but prolonged the bleeding and clotting times. The vasodilators (histamine and depropanex) did not affect tumor growth; in these mice and in the untreated controls the weights of the tumors ranged from 7 to 16 gm. (av. 10.9 gm.).

2. In the suramin-treated mice the tumors weighed 1 to 7 gm. (av. 3.5) and the inhibition of their growth apparently resulted from the necrosis of neoplastic lymphocytes, but morphologically normal tumor cells were still present. In the lymph nodes and spleens of these animals there was an obvious diminution in the number of lymphocytes, the usual lymphoid areas of these organs consisting almost entirely of reticular cells, fibroblasts, large lymphocytes, and plasma cells. The lymph nodes showed much less actual necrosis of lymphoid elements *in situ* than did the tumors. Bleeding and clotting times were prolonged, and in most cases there was renal damage.

3. Tissue from 2 tumors that grew in suramin-treated animals was successfully transplanted to 10 other C3H mice, thus demonstrating the presence of viable neoplastic cells.

4. Smaller doses of suramin given immediately after transplantation did not significantly inhibit the eventual growth of the tumor.

REFERENCES

1. Brazda, F., Williams, W. L., and Wiedorn, M. S. The Effects of Suramin (Germanin) on the Respiration and Morphology of Lymphoid Tissues of the Rat. (To be published.)
2. Brues, A. M., and Cohen, A. Effects of Colchicine and Related Substances on Cell Division. Biochem. J., **30**: 1363-1368. 1936.
3. Brues, A. M., and Marble, B. B. Lymphoblastoma in Mice Following Administration of Carcinogenic Tar. Am. J. Cancer, **37**:45-53. 1939.
4. Chambers, R., Cameron, Gladys, and Kopac, M. J. Neoplasm Studies. XI. The Effects in Tissue Culture of N, N, N′, N′-Tetramethyl-*o*-Phenylenediamine and Other Compounds on Malignant Lymph Nodes. Cancer Research, **3**:293-295. 1943.
5. Dougherty, T. F., Williams, W. L., and Gardner, W. U. Changes in Myeloid and Lymphoid Tissues in Estrogen Treated Dogs. Anat. Rec., **85**:19, Suppl. 1943.
6. Dougherty, T. F., and White, A. Effect of Pituitary Adrenotropic Hormone on Lymphoid Tissue. Proc. Soc. Exper. Biol. & Med., **53**:132-133. 1943.
7. Dougherty, T. F., and White, A. Influence of Adrenal Cortical Secretion on Blood Elements. Science, **98**:367-369. 1943.
8. Drinker, C. K., and Joffey, J. M. Lymphatics, Lymph, and Lymphoid Tissue. Cambridge: Harvard University Press. 1941.
9. Dunlap, C. E. Effects of Radiation on the Blood and Hemopoietic Tissues, Including the Spleen, Thymus and the Lymph Nodes. *In* Warren, S., Effects of Radiation on Normal Tissues pt. 3, General Review in Arch. Path., **34**:562-608. 1942.
10. Edmunds, C. W., and Gunn, J. A. (Editors). Cushny's Pharmacology and Therapeutics. Philadelphia: Lea & Febiger. 1940.
11. Engelbreth-Holm, J. Leukemia in Animals. London: Oliver & Boyd. 1942.
12. Engelbreth-Holm, J., and Lefèvre, H. Acceleration of the Development of Leukemias and Mammary Carcinomas in Mice by 9,10-Dimethyl-1,2-Benzanthracene. Cancer Research, **1**:102-108. 1941.
13. Flory, C. M., Furth, J., Saxton, J. A., Jr., and Reiner, L. Chemotherapeutic Studies on Transmitted Mouse Leukemia. Cancer Research, **3**:729-743. 1943.
14. Forkner, C. E. Leukemia and Allied Disorders. New York: Macmillan Company. 1938.
15. Furth, J., and Barnes, W. A. Differences between Malignant Blood Cells from Induced and Spontaneous Leukemias of Mice. Cancer Research, **1**:17-22. 1941.
16. Furth, J., and Furth, O. B. Neoplastic Diseases Produced in Mice by General Irradiation with X-Rays. I. Incidence and Types of Neoplasms. Am. J. Cancer, **28**:54-65. 1936.
17. Gardner, W. U. Tumors in Experimental Animals Receiving Steroid Hormones. Surgery, **16**:8-32. 1944.
18. Gardner, W. U., and Dougherty, T. F. The Leukemogenic Action of Estrogens in Hybrid Mice. Yale J. Biol. & Med., **17**:75-90. 1944.
19. Gardner, W. U., Dougherty, T. F., and Williams, W. L. Lymphoid Tumors in Mice Receiving Steroid Hormones. Cancer Research, **4**:73-87. 1944.
20. Gardner, W. U., Kirschbaum, A., and Strong, L. C. Lymphoid Tumors in Mice Receiving Estrogens. Arch. Path., **29**:1-7. 1940.
21. Goodman, L., and Gilman, A. The Pharmacological Basis of Therapeutics. New York: The Macmillan Company. 1941.
22. Gortner, R. A. Outlines of Biochemistry. New York: John Wiley and Sons. 1938.
23. Henshaw, P. S., Thompson, J. W., and Meyer, H. L. Experimental Roentgen Injury. V. Effects on Hematopoietic Reserves and Regenerative Capacity. J. Nat. Cancer Inst., **5**:233-247. 1945.
24. Heilman, F. R., and Kendall, E. C. Influence of 11-Dehydro-17-Hydroxycorticosterone (Compound E) on Growth of a Malignant Tumor in the Mouse. Endocrinology, **34**:416-420. 1944.
25. Hoch-Ligeti, Cornelia. Studies on the Changes in the Lymphoid Tissue of Mice Treated with Carcinogenic and Noncarcinogenic Hydrocarbons. Cancer Research, **1**:484-488. 1941.
26. Humphreys, E. M., and Donaldson, L. Degeneration of the Adrenal Cortex Produced by Germanin. Am. J. Path., **17**:767-776. 1941.
27. Jackson, C. M. The Effects of Inanition and Malnutrition upon Growth and Structure. Philadelphia: P. Blakiston. 1925.

28. Jackson, D. E. Experimental Pharmacology and Materia Medica. St. Louis: C. V. Mosby Company. 1939.
29. Kirschbaum, A. Recent Studies on Experimental Mammalian Leukemia. Yale J. Biol. & Med., **17**:163-187. 1944.
30. Kirschbaum, A., Strong, L. C., and Gardner, W. U. Influence of Methylcholanthrene on Age Incidence of Leukemia in Several Strains of Mice. Proc. Soc. Exper. Biol. & Med., **45**:287-289. 1940.
31. Kirschbaum, A., and Strong, L. C. Influence of Carcinogens on the Age Incidence of Leukemia in the High Leukemia F Strain of Mice. Cancer Research, **2**:841-845. 1942.
32. Krebs, C., Rask-Nielsen, H. C., and Wagner, A. The Origin of Lymphosarcomatosis and Its Relation to Other Forms of Leucosis in White Mice. Acta radiol., suppl. **10**:1-53. 1930.
33. Lacassagne, A. Sarcomes Lymphoïdes apparus chez des Souris longuement traiteés par des hormones oestrogènes. Comp. rend. Soc. de biol., **126**:193. 1937.
34. Law, L. W. The Induction of Leukemia in Mice Following Percutaneous Application of 9,10-Dimethyl-1,2-Benzanthracene. Cancer Research, **1**:564-571. 1941.
35. Lits, F. J. Recherches sur les Réactions Cellulaires Provoqueés par la Colchicine. Arch. Internat. de Méd. Expér., **11**:811-901. 1936.
36. Lits, F. J., Kirschbaum, A., and Strong, L. C. Action of Colchicine on a Transplanted Malignant Neoplasm in Mice of the C3H Strain. Am. J. Cancer, **34**:196-213. 1938.
37. Ludford, R. J. Action of Toxic Substances upon Division of Normal and Malignant Cells *in Vitro* and *in Vivo*. Arch. f. exper. Zellforsch., **18**:411-441. 1936.
38. McEndy, D. P., Boon, Mary C., and Furth, J. Induction of Leukemia in Mice by Methylcholanthrene and X-Rays. J. Nat. Cancer Inst., **3**:227-247. 1942.
39. McEndy, D. P., Boon, Mary C., and Furth, J. On the Role of Thymus, Spleen, and Gonads in the Development of Leukemia in a High-Leukemia Stock of Mice. Cancer Research, **4**:377-383. 1944.
40. McGuigan, H. A. Applied Pharmacology. St. Louis: Mosby. 1940.
41. Mider, G. B., and Morton, J. J. The Effect of Methylcholanthrene on the Latent Period of Lymphomatosis in Dilute Brown Mice. Am. J. Cancer, **37**:355-363. 1939.
42. Moon, H. D. Effect of Adrenocorticotropic Hormone in 4-Day-Old Rats. Proc. Soc. Exper. Biol. & Med., **43**: 42-44. 1940.
43. Morton, J. J., and Mider, G. B. Some Effects of Carcinogenic Agents on Mice Subject to Spontaneous Leukoses. Cancer Research, **1**:95-98. 1941.
44. Murphy, J. B., The Lymphocyte in Resistance to Tissue Grafting, Malignant Disease, and Tuberculous Infection. Monographs of the Rockefeller Institute for Medical Research, No. 21. 1926.
45. Murphy, J. B. The Effect of Castration, Theelin, and Testosterone on the Incidence of Leukemia in a Rockefeller Institute Strain of Mice. Cancer Research, **4**:622-624. 1944.
46. Murphy, J. B., and Sturm, E. The Effect of Adrenal Cortical and Pituitary Adrenotropic Hormones on Transplanted Leukemia in Rats. Science, **99**:303. 1944.
47. Parsons, L. D., and Warren, F. L. Cellular Changes in the Spleen and Lymph Glands in Mice Used for Carcinogenic and Related Experiments, with Special Reference to the Giant Cells of the Spleen. J. Path. & Bact., **52**:305-321. 1941.
48. Perry, I. H., and Ginzton, L. L. The Development of Tumors in Female Mice Treated with 1:2:5:6-Dibenzanthracene and Theelin. Am. J. Cancer, **29**:680-704. 1937.
49. Quick, A. J. The Hemorrhagic Diseases and the Physiology of Hemostasis. Baltimore: Charles C. Thomas. 1942.
50. Rous, P., Gilding, H. P., and Smith, F. The Gradient of Vascular Permeability. J. Exper. Med., **51**:807-830. 1930.
51. Schacher, J., Browne, J. S. L., and Selye, H. Effects of Various Sterols on Thymus in Adrenalectomized Rat. Proc. Soc. Exper. Biol. & Med., **36**:488-491. 1936.
52. Sollman, T. A. Manual of Pharmacology. Philadelphia and London: W. B. Saunders Company. 1942.
53. Tomlinson, C. C., and Cameron, O. J. Juvenile Pemphigus, Effects of Germanin in Three Cases. Arch. Dermat. & Syph., **38**:555-568. 1938.
54. Tyslowitz, R., and Dingemanse, E. Effect of Large Doses of Estrogen on the Blood Picture of Dogs. Endocrinology, **29**:817-827. 1941.
55. Williams, W. L. The Effects of Gastric Mucin on the Growth of Transplanted Tumor (Lymphosarcoma) Cells in Mice. Yale J. Biol. & Med., **17**:311-318. 1944.
56. Williams, W. L., and Cunningham, B. Histological Changes in the Rat Testis Following Heat Treatment. Yale J. Biol. & Med., **12**:309-316. 1939.
57. Williams, W. L., and Hodge, H. C. Intra Vitam Staining and Toxicity of Chlorazol Fast Pink in Mice and Rats. Anat. Rec., **87**:181-209. 1943.
58. Williams, W. L., Stein, Kathryn F., and Allen, E. Reaction of Genital Tissues of the Female Mouse to the Local Application of Colchicine. Yale J. Biol. & Med., **13**:841-846. 1941.

Induction of Mammary Cancer with Methylcholanthrene

I. Histogenesis of the Induced Neoplasm*

Arthur Kirschbaum, Ph.D., M.D., W. Lane Williams, Ph.D., and John J. Bittner, Ph.D.

(Department of Anatomy, and Division of Cancer Biology, Department of Physiology, University of Minnesota Medical School, Minneapolis 14, Minnesota)

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What is the relationship of the neoplasm induced by carcinogenic hydrocarbons to the spontaneous malignant tumor? Neoplasms (epidermoid carcinoma, myxosarcoma, and fibrosarcoma) that never appear spontaneously in certain strains of mice can be induced readily by the action of carcinogens such as methylcholanthrene. Carcinogens would seem, however, to potentiate spontaneous mechanisms of carcinogenesis, as is demonstrated by the accelerated onset of certain neoplasms—leukemia (2), mammary cancer (1, 8), lung tumors (1), hepatomas (24)—in genetically susceptible strains following administration of these agents.

Does experimental chemical carcinogenesis involve the institution of a different chain of events, or augmentation of the same mechanism that produces the spontaneous tumor? If the same normal parent tissue is the source of both, microscopic structure and histogenesis would be of importance in any comparison of spontaneous and induced neoplasms. Such observations can be made readily in the case of spontaneous and induced cancers of the mouse mammary gland.

Mammary cancer appears spontaneously in female mice of the dba strain, but developed much earlier when the skin was painted with methylcholanthrene dissolved in benzene (9, 8, 18). Not only were breeders more susceptible than virgins with respect to total incidence and rapidity of onset; the number of induced tumors was greater, also, and multiple cancerous and precancerous nodules appeared in painted and forced-bred females of this strain (sublines 12 and 212), with transitions in size from minute nodules to frank cancer (18). The cancers were within the mammary glands, were freely movable, and could be distinguished readily from skin tumors.

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Although three factors, genetic susceptibility, hormonal stimulation of mammary growth, and the milk agent, were found necessary for the development of spontaneous mammary cancer in inbred strains with a high incidence of the disease (2), two of these, the milk agent (17) and a genetic susceptibility to the development of spontaneous mammary cancer (23), were not essential for the development of induced mammary cancer. There was suggestive evidence, however, that the milk agent may influence the course of development of mammary tumors induced by methylcholanthrene (17). Hormonal stimulation of the mammary gland seemed essential in conditioning its neoplastic response to methylcholanthrene, since only females developed the induced disease.

The purpose of this report is to present an account of the histogenesis of methylcholanthrene mammary cancer, and to compare this process with the histological development of spontaneous mammary cancer in the mouse.

MATERIALS AND METHODS

Table I indicates the genetic type, treatment, and number of animals in each group studied. The dba and A stocks are high mammary cancer strains. Strains Z and Zb are genetically identical; Z mice possess the milk agent and develop mammary cancer spontaneously, whereas Zb animals lack the milk agent and do not develop mammary cancer. Stocks C57, NH, and F are low mammary cancer strains without the milk agent.

Methylcholanthrene (0.25 per cent solution in benzene) was applied by painting a different area of the skin 3 times a week; the sequence of sites was the back, each of the legs, and the axillary and inguinal regions. The applications were continued until the animals developed a neoplasm (mammary cancer, skin cancer, or leukemia) that interfered with a relatively healthful existence. Female mice were forced-bred, the young being removed within 48 hours after birth,

and painting was begun when the animals were 5 to 8 weeks of age. All material for histological study was derived from animals that were killed for this purpose; material from animals that had died was used only for gross diagnosis.

Mice were skinned at autopsy, and the skin with the mammary glands was stretched on a cork board and fixed in a modified Lavdovsky's fluid (26). The glands were stained in Harris' hematoxylin and cleared (cedar oil) *in toto*. In some instances portions were embedded in paraffin and sections for microscopic study were prepared from them. Most of the sections were made from mammary glands that had been freed from the skin before fixation; these were fixed in Zenker's fluid while flat and adherent to a piece of paper toweling. Glands were sectioned in a plane tangential to the skin. Portions of the larger tumors were removed for microscopic study; some nodules were excised and fixed individually. After Zenker fixation (4 to 8 hours) the tissue was washed overnight, then placed in 80 per cent alcohol for 15 minutes, in 95 per cent alcohol for 45 minutes, and for a total of 2 hours in 2 changes of acetone; paraffin infiltration and embedding followed. Sections were cut at 6 micra and stained with Delafield's hematoxylin and eosin.

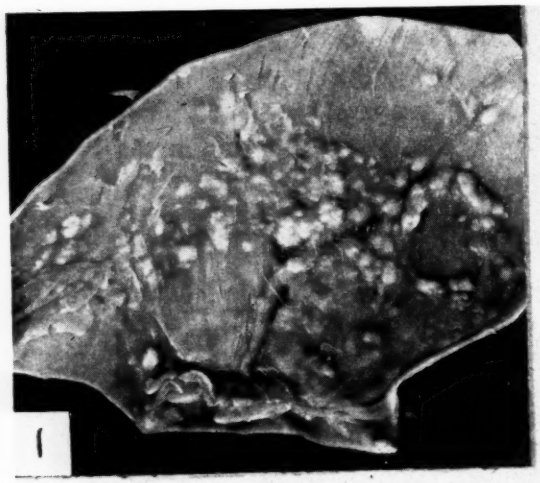
TABLE I: DATA ON CONTROL AND TEST MICE

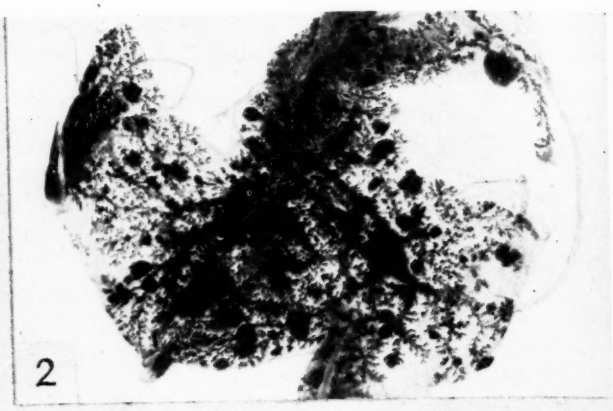
Lineage	Group	No. of mice	Treatment	No. with nodules	No. with mammary cancer	Mean cancer age
		MICE WITHOUT THE MILK AGENT				
* Zb	Breeding ♀♀	123	none	0	0	——
Zb	" ♀♀	24	methylchol.	14	4	209 days
† Zb.D (F_1 hyb.)	Virgin ♀♀	16	"	0	0	——
Zb.d (F_1 ")	Breeding ♀♀	11	"	7	0	——
Zb.d (F_1 ")	Virgin ♀♀	13	"	0	0	——
Zb.d (F_1 ")	Breeding ♀♀	13	"	5	2	308 days
Zbd.d (B Cross)	Virgin ♀♀	5	"	1	1	260 "
Zbd.d (")	Breeding ♀♀	17	"	11	5	257 "
* NH	" ♀♀	60	none	0	0	——
NH.D (F_1 hyb.)	" ♀♀	12	methylchol.	10	2	320 "
$(NH.D)^2$ (F_2 hyb.)	" ♀♀	15	"	12	8	218 "
* C57	" ♀♀	90	none	0	0	——
C57	" ♀♀	43	methylchol.	0	0	——
		MICE WITH THE MILK AGENT				
Z	Breeding ♀♀	224	none	0	213	——
Z	Virgin ♀♀	11	methylchol.	0	0	——
Z	Breeding ♀♀	10	"	7	6	205 days
Z.D (F_1 hyb.)	Virgin ♀♀	4	"	0	0	——
Z.D (")	Breeding ♀♀	4	"	4	4	192 "
Z.d (")	Virgin ♀♀	5	"	0	1	246 "
Z.d (")	Breeding ♀♀	6	"	6	5	215 "
dba (12 & 212)	Virgin ♀♀	59	none	0	29	507 "
dba (")	" ♀♀	43	methylchol.	0	7	240 "
dba (")	Breeding ♀♀	88	none	0	76	341 "
dba (")	" ♀♀	27	methylchol.	27	27	155 "
dba (")	Males	48	"	0	0	——
d.Zbd (B Cross)	Breeding ♀♀	7	"	6	3	218 "
D.NH (F_1 hyb.)	" ♀♀	7	"	7	5	281 "
$(D.NH)_2$ (F_2 ")	" ♀♀	12	"	11	11	222 "
NH.F (F_1 ")	" ♀♀	4	"	3	0	——
A	" ♀♀	128	none	0	11	——
A	" ♀♀	10	methylchol.	2	7	240 "
D.A (F_1 hyb.)	" ♀♀	2	"	2	2	180 "
A.D (")	" ♀♀	7	"	6	4	255 "
$(A.D)^2$ (F_2 ")	" ♀♀	4	"	4	4	166 "
$(D.A)^2$ (")	" ♀♀	6	"	6	6	154 "
DA.B (B Cross)	" ♀♀	10	"	9	6	172 "

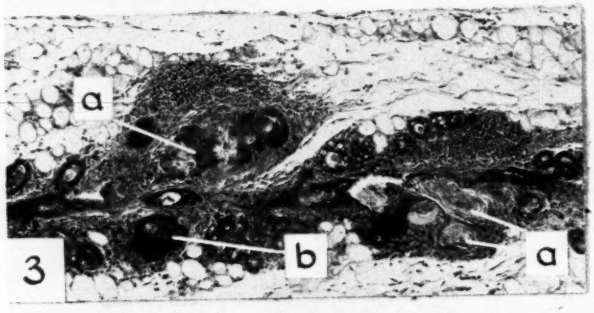
* These stocks and the F stock lack the milk agent.
† D refers to subline 212—strain dba.
d " " " 12—strain dba.

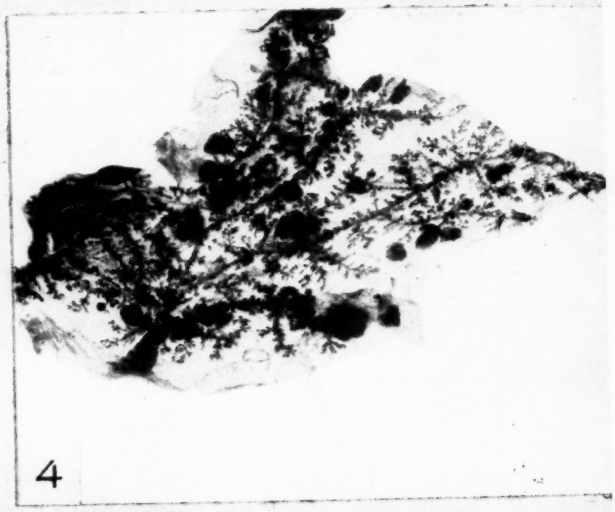
OBSERVATIONS

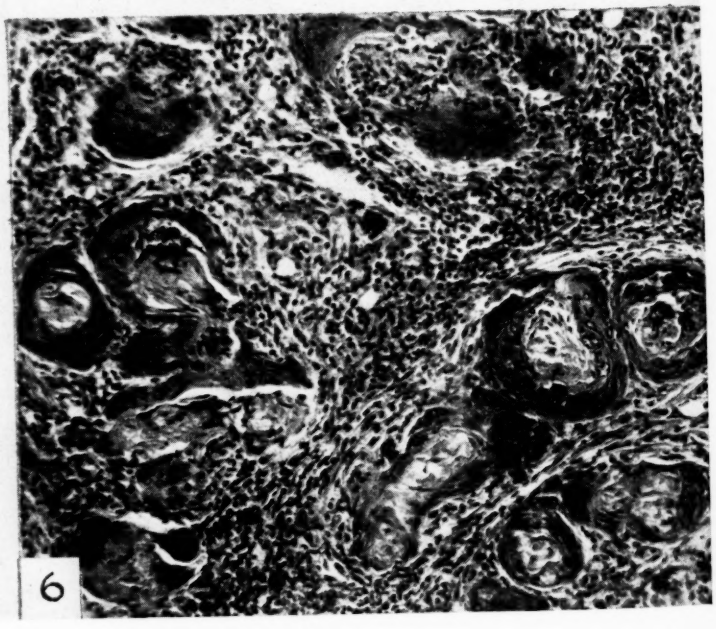
Within 3 to 9 months after methylcholanthrene painting had been started, a majority of the forced-bred females of certain genetic types had developed multiple nodules in most of the mammary glands (Table I, Figs. 1, 2, and 4). Methylcholanthrene-painted, forced-bred C57 females developed neither











FIGS. 1-6

mammary nodules nor carcinomas, indicating that certain stocks are resistant to the carcinogenic induction of breast tumors. The incidence of frank carcinoma of the mammary gland in animals genetically susceptible to the action of methylcholanthrene was higher if the mice possessed the milk agent (Table I).

The nodules represented alterations in the ends of the ducts (Figs. 2, 3, and 4). In whole-mount preparations they resembled the nodules of the mammary tree of untreated breeding dba mice with the milk agent (Figs. 2, 4, and 16). They were more numerous, however, and study of the whole-mount preparations revealed squamous areas that are not present in the hyperplastic adenomatous nodules of the mammary glands from high mammary cancer strains (9). Sections of these nodules demonstrated the presence of stratified squamous, keratinized epithelium (Figs. 3, 5, 6, and 7), an alteration that preceded the true neoplastic change (Fig. 8).

Although formation of the hyperplastic, adenomatous nodule is not induced by methylcholanthrene, the hyperplastic nodule may serve as the site of action of methylcholanthrene. Fig. 15 demonstrates keratinization of ductal epithelium within a hyperplastic nodule following 18 paintings with the carcinogen.

The sequence of events in nodule formation appeared to be the following. First, injury was produced by the carcinogen, as demonstrated by epithelial sloughing within ducts and proximal alveoli (Figs. 3, 5, and 9). Second, there was a proliferative epithelial response to carcinogenic injury characterized by squamous metaplasia (Figs. 6 and 7). Third, neoplastic alteration of the modified epithelium took place while the growth was still a nodule (Figs. 5 and 9). There was an accompanying inflammatory cellular infiltration in the surrounding connective tissue (Fig. 6).

The cancerous nodules grew progressively to produce palpable cancers in hosts that lived sufficiently long. Histologically these mammary cancers varied in structure, although there was usually a squamous component (Figs. 11 and 13). Most tumors possessed some cancerous tissue with an alveolar arrangement (Figs. 11 and 12). The cells of the basal layer of the squamous epithelium within the cancers seemed capable of giving rise to alveolar cancer (Fig. 11).

As a result of forced breeding alone, neither multiple nodules nor mammary cancer developed in either Zb females or hybrid females with an NH maternal contribution (genetically comparable to carcinogen-treated, "without the milk agent" mice listed in Table I). Not a single case of mammary cancer has been seen in over 100 breeding NH female mice that lived to be more than 1 year of age.

Mammary cancer appeared earlier in painted mice with the milk agent (Table I) than in genetically similar mice lacking the agent. There was histologic evidence for the carcinogenic induction of these cancers (Figs. 3 and 10).

DISCUSSION

In contrast to estrogenic hormone (4), methylcholanthrene induces the development of mammary cancer in mice in the absence of the milk agent. Hormonal stimulation of mammary growth is necessary, however, for methylcholanthrene mammary carcinogenesis. The single etiologic factor common to both methylcholanthrene and milk agent mammary cancer is hormonal stimulation of the mammary gland. The activity of the carcinogenic hydrocarbons in mammary carcinogenesis would seem not to be mediated by the only known virus-like agent concerned in the spontaneous genesis of mammary cancer.

The carcinogen-induced nodules were precancerous lesions, for there was both gross and microscopic evidence for transformation to carcinoma. Histologically the nodules differed from the hyperplastic nodules that are thought to represent the precancerous lesion for spontaneous mouse mammary cancer (14, 15). The latter are characterized by clusters of alveoli that resemble lactating alveoli (Fig. 18) and are found in glands otherwise inactive from the secretory standpoint. The carcinogen-induced nodules were areas in

DESCRIPTION OF FIGURES 1 TO 6

FIG. 1.—Thoracic mammary gland of methylcholanthrene-painted, forced-bred F_2 hybrid (NH.D) female showing multiple nodules. Gland fixed *in toto* on stretched skin. Mag. $\times 2$.

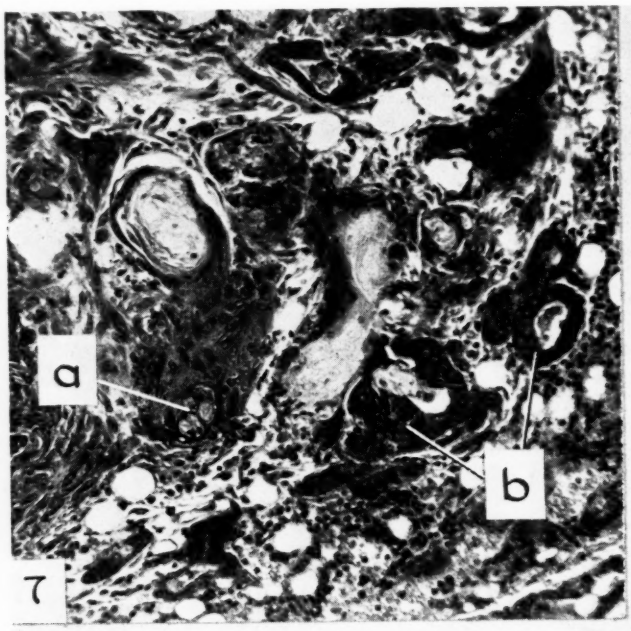
FIG. 2.—Whole mount of thoracic mammary gland of methylcholanthrene-painted, forced-bred F_2 hybrid (NH.D) female. Note numerous nodules. Mag. $\times 3$.

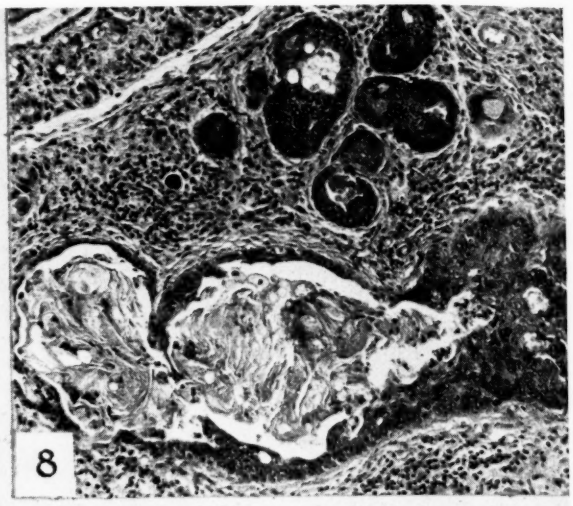
FIG. 3.—Longitudinal section through duct and proximal alveoli of mammary gland of forced-bred (Z) female. Note squamous metaplasia (a), hyperplasia (b) of epithelium of duct and alveoli, and infiltration of surrounding connective tissue with leukocytes. Mag. $\times 63$.

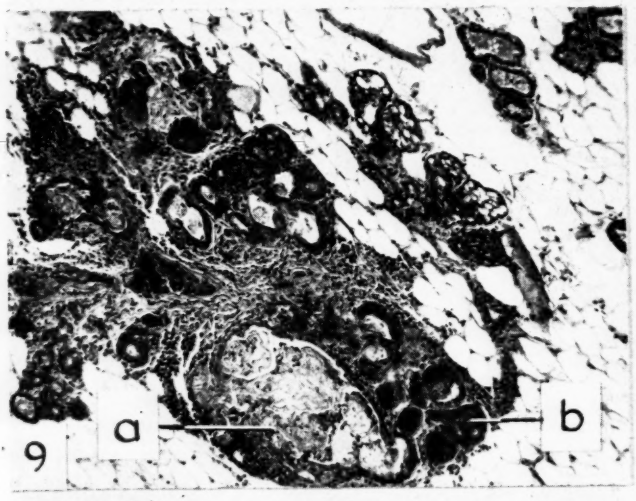
FIG. 4.—Whole mount of thoracic mammary gland of methylcholanthrene-painted, forced-bred F_2 hybrid (D.NH) female. Note numerous nodules. Mag. $\times 3$.

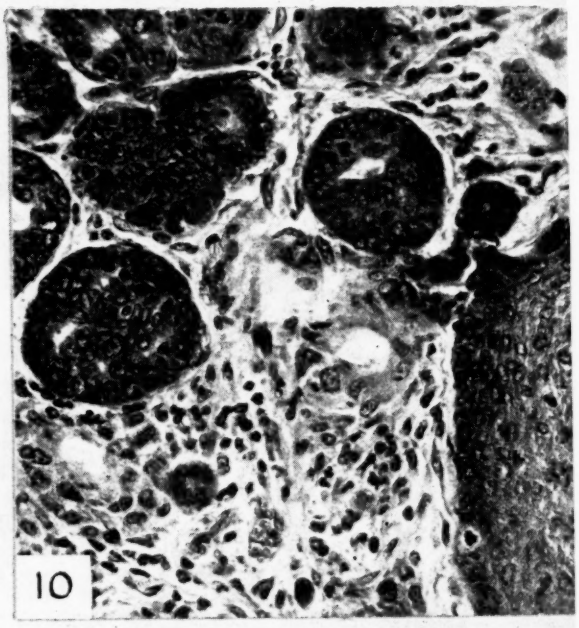
FIG. 5.—Section of carcinogen-induced nodule from mammary gland of forced-bred Zb female, showing extensive squamous metaplasia and area of adenocarcinoma (upper left). Mag. $\times 90$.

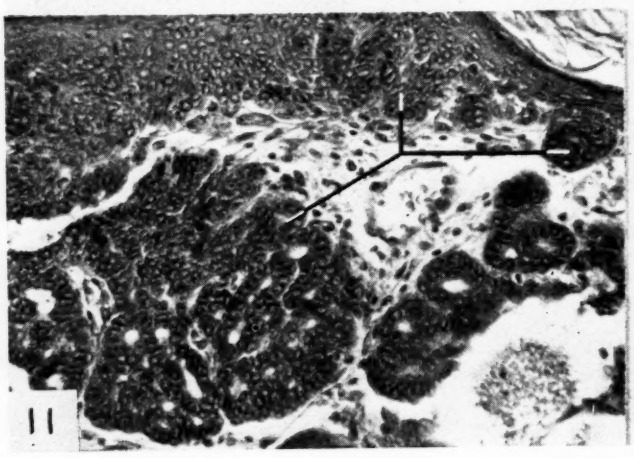
FIG. 6.—Section through carcinogen-induced nodule, showing squamous metaplasia of small ducts and leukocytic infiltration. Mag. $\times 175$.

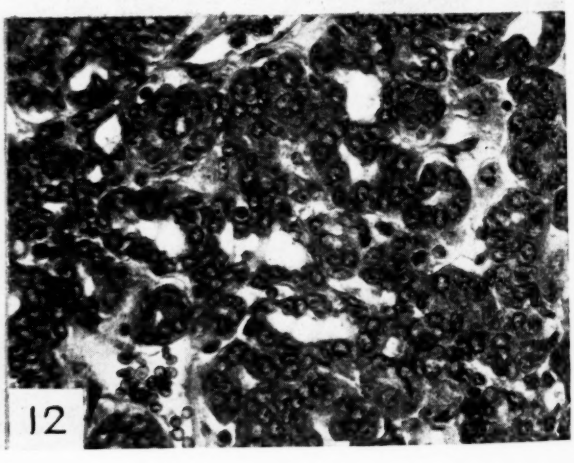












FIGS. 7-12

which the epithelium of the ducts and proximal alveoli had undergone squamous metaplasia, probably as a response to injury, for the surrounding connective tissue was infiltrated with leukocytes. Carcinoma arose from nonkeratinized epithelium of the nodules. In some nodules an extremely small amount of ductal epithelium remained uninjured.

Thus the evidence demonstrates that the histogenesis of methylcholanthrene tumors differs from that of the usual spontaneous mammary tumors of mice possessing the milk agent. The major difference is the prevalence of squamous metaplasia of ductal epithelium during the development of the former. Some degree of squamous metaplasia was still evident in the large tumors that developed in the painted mice (Fig. 13). However, large areas of a given methylcholanthrene tumor were morphologically identical with the typical spontaneous mammary adenocarcinomas of mice (Fig. 12).

Squamous metaplasia has previously been observed in carcinogen-induced mammary tumors (6, 19, 22, 25). So-called hyperplastic nodules with squamous metaplasia (Fig. 17) occur spontaneously, particularly in the mammary glands of forced-bred female mice without the milk agent (13). Although spontaneous mammary adenocarcinoma appears infrequently in female mice lacking the milk agent, forced-bred F_1 hybrid female mice without this agent develop slowly growing mammary tumors with extensive squamous metaplasia at an age of 1,000 days or more (12). This type of spontaneous mammary tumor bears a greater resemblance to the carcinogen-induced neoplasm than does the milk agent tumor.

The incidence of mammary cancer in carcinogen-treated mice of certain genetic types was higher if the females possessed the milk agent. The fact that tumors in many carcinogen-treated mice with the agent were multiple suggests that these were induced, rather than entirely independent of the carcinogenic hydrocarbon. The histogenesis of methylcholanthrene mammary cancer was the same in either the presence or absence of the milk agent.

The difference in histogenesis of the spontaneous milk agent tumor and the carcinogen-induced mammary tumor might suggest that the processes are unrelated, and that the carcinogen does not augment a spontaneous mechanism. However, if the milk agent actually does potentiate the carcinogenic activity of methylcholanthrene, then the two processes might be considered analagous. If the milk agent tumors and the carcinogen-induced tumors are to be etiologically associated, it might be supposed that the milk agent produces a specific type of injury within the mammary gland, the response to which is the hyperplastic adenomatous nodule.

The histogenesis of carcinogen-induced mammary cancer is comparable in its stages to the development of skin cancer following the application of a carcinogen. In both cases there is an insult to the tissue, followed by hyperplastic repair and then neoplasia (4). There are many examples of carcinogenesis following specific injury—for example, skin cancer after x-ray burns or prolonged exposure to ultraviolet rays, leukemia in mice following damage to the lymphoid cells by x-rays (16), and carcinoma in a cirrhotic liver previously damaged by the administration of carbon tetrachloride (7).

It has been suggested that the carcinogen itself is not the growth promoting agent, but that the latter is formed in the tissue after a specific type of damage (5). It is possible, although there is evidence to the contrary, that the milk agent is an excitant of neoplasia that is derived from damaged tissue. The carcinogen-induced tumors have not been tested for the milk agent; it would be of great interest if a carcinogen were responsible for the appearance of this agent in mice that did not possess it at the inception of the experiment.

Methylcholanthrene may substitute for the milk agent when mammary cancer is induced in the absence of the latter. On the basis of such an interpretation the milk agent would then be considered the "natural carcinogen."

Carcinogen-induced leukemias of hybrid mice are genetically different from leukemias occurring spontaneously in the same types of hybrid animals (10). This was demonstrated by transplantation experiments, and suggests that the carcinogen might not

DESCRIPTION OF FIGURES 7 TO 12

FIG. 7.—Carcinogen-induced nodule of Z forced-bred female, showing squamous metaplasia (a) and areas of early carcinoma (b). Mag. × 175.

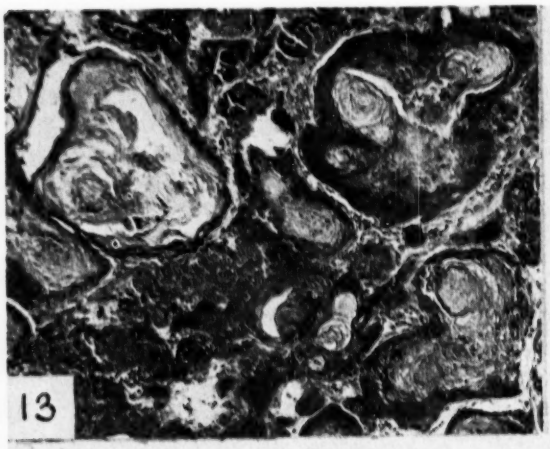
FIG. 8.—Development of adenocarcinoma from metaplastic squamous epithelium of methylcholanthrene-painted forced-bred F_1 hybrid (ZB.D) female. Mag. × 150.

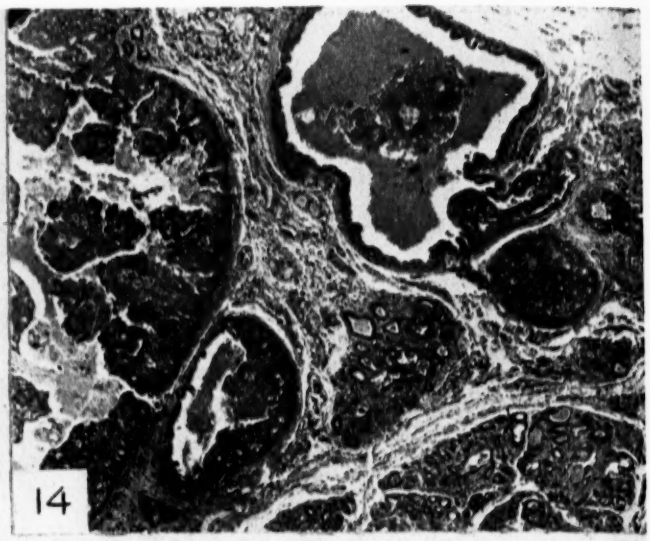
FIG. 9.—Squamous metaplasia (a) and early carcinoma (b) in mammary nodule from methylcholanthrene-painted, forced-bred Z female. Mag. × 90.

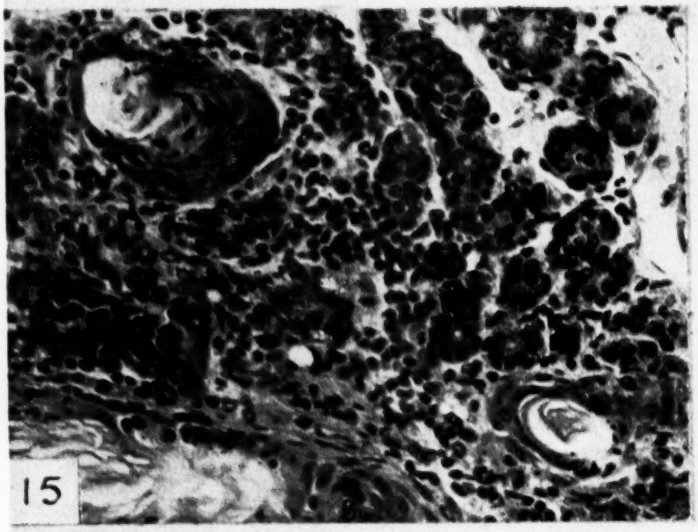
FIG. 10.—Early carcinoma and adjacent duct lined by metaplastic squamous epithelium. From carcinogen-painted, forced-bred Z female. Mag. × 325.

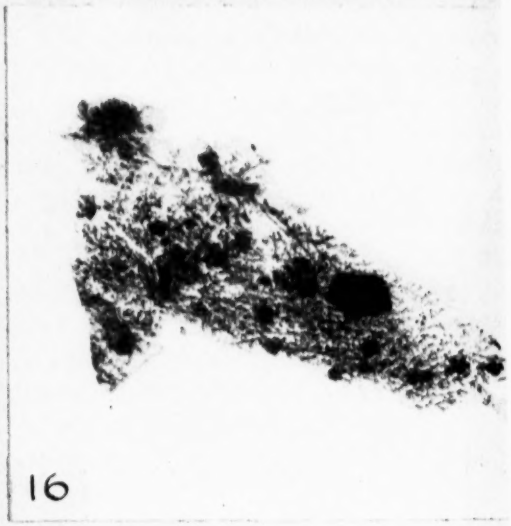
FIG. 11.—Carcinogen-induced mammary cancer, illustrating differentiation of alveoli from basal layer of squamous epithelium. Mag. × 225.

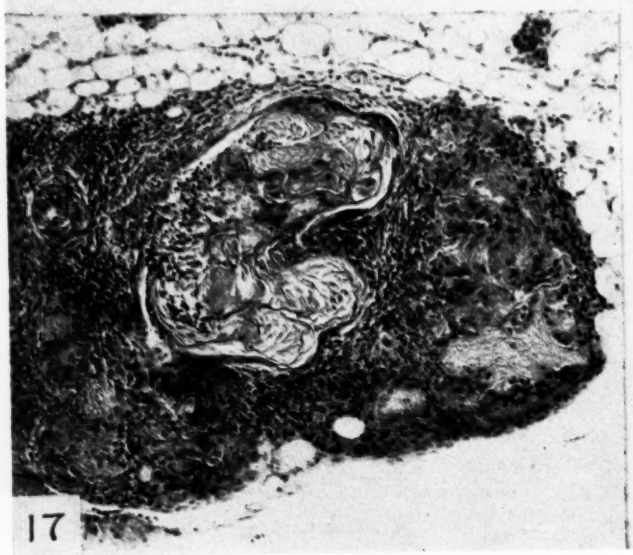
FIG. 12.—Area from carcinogen-induced mammary cancer, showing structure typical of spontaneous mouse adenocarcinoma. Mag. × 300.

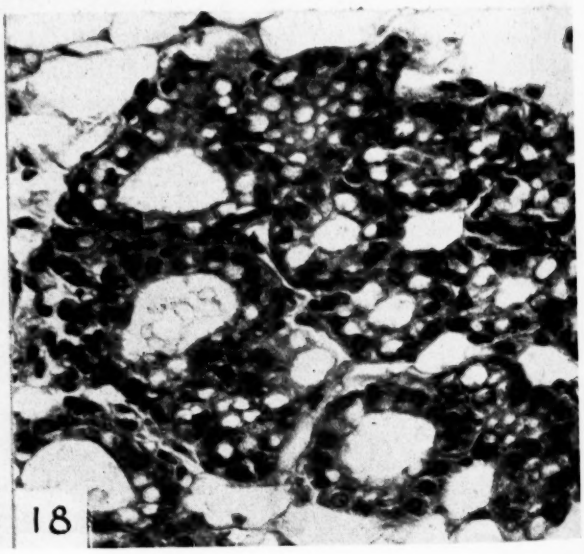












FIGS. 13-18

have been accelerating the spontaneous mechanism. However, methylcholanthrene induced an early appearance of either myeloid or lymphatic leukemia in strain C mice (20), which develop both types of leukemia spontaneously, whereas in stocks that develop only lymphatic leukemia spontaneously this type of leukemia alone could be induced by either carcinogens, x-rays, or estrogens.

If under experimental conditions different etiologic mechanisms can be demonstrated for different histological types of cancer of the same tissue, as in mouse mammary cancer, it would seem likely that different etiological mechanisms might be involved in spontaneous carcinogenesis of tumors of the same tissue. For example, each of the different types of spontaneous mammary cancer of the human subject might be etiologically independent.

Certain stocks of mice exhibit a greater genetic response than others to the induction of mammary cancer with methylcholanthrene. The dba strain is extremely susceptible, whereas other high-cancer strains (C3H, A) do not respond so readily. Although neither the NH nor the C57 strain of mice develops spontaneous mammary cancer, one (C57) was resistant to the carcinogenic induction of breast tumors, whereas the other (NH) was highly susceptible. This suggests that susceptibility to induced mammary cancer may not be of the same nature as susceptibility to spontaneous mammary cancer.

SUMMARY

When painted with methylcholanthrene dissolved in benzene, forced-bred female mice of certain genetic types developed grossly visible, multiple nodules in the mammary gland. Forced breeding alone did not induce this type of nodule formation. These nodules, which developed in either the presence or absence of the milk agent, are thought to have been induced by the carcinogen. Although in whole-mount preparations viewed under low magnification the nodules resembled the hyperplastic nodules that precede milk agent tumors, sections revealed under higher powers a decidedly different histology. The nodules developed as a result of injury to the epithelium of the ducts and proximal alveoli by the carcinogen, followed by a proliferative squamous epithelial response. These alterations were succeeded by neoplasia, and the frank cancers that developed were of mixed squamous and alveolar structure. Susceptibility to the carcinogenic induction of mammary cancer is not common to all strains, and this susceptibility cannot be correlated with susceptibility to spontaneous mammary cancer. The histological evidence does not favor the concept that the carcinogen accelerates the sequence of alterations seen in the histogenesis of spontaneous milk agent tumors of mice.

REFERENCES

1. Andervont, H. B. Pulmonary Tumors in Mice. Pub. Health Rep., **54**:1512-1533. 1939.
2. Bittner, J. J. "Influences" of Breast-Cancer Development in Mice. Pub. Health Rep., **54**:1590-1597. 1939.
3. Bittner, J. J. The Influence of Estrogens on the Incidence of Tumors in Foster Nursed Mice. Cancer Research, **1**:290-292. 1941.
4. Cramer, W., and Stowell, R. E. The Early Stages of Carcinogenesis by 20-Methylcholanthrene in the Skin of the Mouse. I. Experimental Technique and Macroscopic Changes. J. Nat. Cancer Inst., **2**:369-378. 1942.
5. Cramer, W., and Stowell, R. E. The Early Stages of Carcinogenesis by 20-Methylcholanthrene in the Skin of the Mouse. II. Microscopic Tissue Changes. J. Nat. Cancer Inst., **2**:379-402. 1942.
6. Dunning, W. F., Curtis, M. R., and Eisen, M. J. The Carcinogenic Activity of Methylcholanthrene in Rats. Am. J. Cancer, **40**:85-127. 1940.
7. Edwards, J. E., and Dalton, A. J. Induction of Cirrhosis of the Liver and of Hepatomas in Mice with Carbon Tetrachloride. J. Nat. Cancer Inst., **3**:19-41. 1942.
8. Engelbreth-Holm, J. Acceleration of the Development of Mammary Carcinoma in Mice by Methylcholanthrene. Cancer Research, **1**:109-112. 1941.
9. Engelbreth-Holm, J., and Lefevre, H. Acceleration of the Development of Leukemias and Mammary Carcinomas in Mice by 9, 10-Dimethyl-1, 2,-Benzanthracene. Cancer Research, **1**:102-108. 1941.
10. Furth, J., and Barnes, W. A. Differences between Malignant Blood Cells from Induced and Spontaneous Leukemias of Mice. Cancer Research, **1**:17-22. 1941.
11. Gardner, W. U., Tumors in Experimental Animals Receiving Steroid Hormones. Surgery, **16**:8-32. 1944.
12. Gardner, W. U. Personal Communication. 1945.
13. Gardner, W. U., and Strong, L. C. The Normal Development of the Mammary Glands of Virgin Females of Ten Strains Varying in Susceptibility to Spontaneous Neoplasms. Am. J. Cancer, **25**:282-290. 1935.

DESCRIPTION OF FIGURES 13 TO 18

Fig. 13.—Portion of large, carcinogen-induced mammary tumor, showing extensive squamous metaplasia as well as numerous alveolar-like components of usual type; cancer arose in a ZB mouse. Mag. × 90.

Fig. 14.—Mammary tumor arising in methylcholanthrene-painted ZB.D F_1 hybrid. Note cysts lined by neoplastic epithelium. Mag. × 120.

Fig. 15.—Squamous metaplasia within a hyperplastic alveolar nodule of a dba mouse painted with carcinogen 18 times. Mag. × 225.

Fig. 16.—Whole mount of untreated dba female breeder, showing typical hyperplastic nodules. Mag. × 3.

Fig. 17.—Nodule from virgin CBA, showing squamous metaplasia. Mag. × 150 (material of Dr. W. U. Gardner).

Fig. 18.—Section showing structure of one of the nodules in whole mount illustrated in Fig. 16. Mag. × 325.

14. Gardner, W. U., Strong, L. C., and Smith, G. M. The Mammary Glands of Mature Female Mice of Strains Varying in Susceptibility to Spontaneous Tumor Development. Am. J. Cancer, **37**:510-517. 1939.

15. Haaland, M. Spontaneous Tumors in Mice. Fourth Scientific Report, Imperial Cancer Research Fund, London, 1911, pp. 1-113.

16. Henshaw, P. S. Experimental Roentgen Injury. IV. Effects of Repeated Small Doses of X-Rays on Blood Picture, Tissue Morphology, and Life Span in Mice. J. Nat. Cancer Inst., **4**:513-522. 1944.

17. Kirschbaum, A., and Bittner, J. J. Relation of the Milk Influence to the Carcinogenic Induction of Mammary Cancer in Mice. Proc. Soc. Exper. Biol. & Med., **58**: 18-19. 1945.

18. Kirschbaum, A., Lawrason, F. D., Kaplan, H. S., and Bittner, J. J. Influence of Breeding on Induction of Mammary Cancer with Methylcholanthrene in Strain Dba Female Mice. Proc. Soc. Exper. Biol. & Med., **55**:141-142. 1944.

19. Kirschbaum, A., and Strong, L. C. Influence of Carcinogens on the Age Incidence of Leukemia in the High Leukemia F Strain of Mice. Cancer Research, **2**:841-845. 1942.

20. Kirschbaum, A., Strong, L. C., and Gardner, W. U. Influence of Methylcholanthrene on Age Incidence of Leukemia in Several Strains of Mice. Proc. Soc. Exper. Biol. & Med., **45**:287-289. 1940.

21. Mider, G. B., and Morton, J. J. The Effect of Methylcholanthrene on the Latent Period of Lymphomatosis in Dilute Brown Mice. Am. J. Cancer, **37**:355-363. 1939.

22. Orr, J. W. Mammary Carcinoma in Mice Following the Intranasal Administration of Methylcholanthrene. J. Path. & Bact., **55**:483-488. 1943.

23. Strong, L. C. X. Carcinoma of Mammary Gland Following Injection of Methylcholanthrene into Mice of NHO Strain. Proc. Soc. Exper. Biol. & Med., **59**:217-220. 1945.

24. Strong, L. C., Smith, G. M., and Gardner, W. U. Induction of Tumors by 3:4:5:6-Dibenzcarbazole in Male Mice of the CBA Strain, Which Develops Spontaneous Hepatomas. Yale J. Biol. & Med., **10**:335-346. 1938.

25. Strong, L. C., and Williams, W. L. A Genetic Analysis of the Induction of Tumors by Methylcholanthrene. III. Local and Remote Induction of Carcinoma of the Mammary Gland. Cancer Research, **1**:886-890. 1941.

26. Williams, W. L., and Hodge, H. C. Intra Vitam Staining and Toxicity of Chlorazol Fast Pink in Mice and Rats. Anat. Rec., **87**:181-209. 1943.

Committee on Growth

Availability of Grants and Fellowships in Cancer Research

The Committee on Growth of the National Research Council, acting for the American Cancer Society, announces that it will entertain applications for Grants in Cancer Research to become effective July 1, 1947. Applications will be received until September 15, 1946.

Applications for Fellowships and Senior Fellowships in Cancer Research of the American Cancer Society for the ensuing year will be received until December 1, 1946.

To date the Committee on Growth has recommended to the American Cancer Society a total of 75 research grants and 14 fellowships. The Committee will continue to recommend support of research in the basic sciences and in clinical investigative medicine broadly pertaining to problems of growth. It will continue also to rely heavily for counsel on its advisory Divisions of Chemistry, Biology, Physics, and Clinical Investigations and their subjacent panels in specialized areas of research.

Applications for research grants for the current year will no longer be entertained. Communications should be addressed to The Committee on Growth, Division of Medical Sciences, National Research Council, 2101 Constitution Avenue, Washington 25, D. C.

Toxin Therapy of Experimental Cancer

The Influence of Protozoan Infections upon Transplanted Cancer

Prof. G. Roskin

[*From the Biological Faculty, University of Moscow, Moscow, U.S.S.R.*]

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Experiments were undertaken to determine the influence of various infections and toxins upon transplanted cancer. After a careful search *Schizotrypanum cruzi* was selected because of its ability to produce a chronic form of schizotrypanosomiasis, chiefly by reason of its organotropism. Our experiments consisted in infecting mice with trypanosomes and inoculating them with the Ehrlich carcinoma. A count of parasites in the peripheral blood and a record of the tumor growth were made every 4 to 5 days. At first a decrease in the development of the infection as well as in that of the tumor occurred in comparison with controls. This was followed by increase in the infection, which finally led to the death of the mouse. The tumor at the same time regressed or disappeared.

The tumors receded in 30 of 45 test animals under the influence of trypanosome infection, while in 15 the growths were inhibited. The grafts grew in all the 45 control mice. In most cases mice simultaneously inoculated with cancer and trypanosomes lived longer than those inoculated with cancer alone, and even longer than the animals inoculated with trypanosomes alone. This experiment, repeated several times with similar results, suggested that trypanosome infection exerts an antagonistic action on mouse cancer.

Another group of mice was infected with relapsing fever (*Sp. duttoni*). The purpose was to determine whether the effect of the *Schizotrypanum cruzi* infection was specific, or whether it was but another form of nonspecific fever therapy. Relapsing fever did not affect the development of grafted tumors.

The third experiment consisted in inoculating 45 guinea pigs with hypernephroma and then infecting them with *Trypanosoma equiperdum*. This form of trypanosomiasis flourishes in guinea pigs, and assumes a chronic form with low mortality. Here trypanosome infection did not reduce the percentage of tumors. The death rate of infected and control guinea pigs with grafted tumors was the same. A slight delay in the appearance and development of these growths in their early stages was noticeable in the infected animals, but retrogression or disappearance of a tumor has never occurred. Spirochetes of relapsing fever and *Trypanosoma equiperdum* differ, therefore, from *Schizotrypanum cruzi*.

Under the influence of *Schizotrypanum* infection, the mouse tumor appeared to melt away. Sections revealed large areas that stained poorly and contained scattered groups of typical cancer cells. Sections from regressing tumors showed accumulations of parasites in the lumina of blood vessels. Many cancer cells contained in their cytoplasm 1 to 3 leishmania-like forms of *Schizotrypanum,* and degenerated or totally disintegrated nuclei.

Extracts of *Schizotrypanum cruzi* had no effect upon cancer cells.

It is possible that the parasite, in disturbing normal metabolism and exhausting the organism, deprives the tumor of substances necessary for its development. Cytologic observations on the tumors of infected mice suggest that trypanosomes reproduce in the cancer cell and eventually cause its degeneration. Tumor cells appear more vulnerable to parasites than normal cells, since in control sections from various normal organs of mice few or no parasites have been found. This suggests that specific toxins may be secreted by *Schizotrypanum cruzi* to which cancer cells may be particularly sensitive.

The Action of *Schizotrypanum* Endotoxin on Tumors

Endotoxins were prepared in the following manner: Heart's blood taken at the height of the disease in a mouse infected with *Schizotrypanum cruzi* was mixed with a 2 per cent sodium citrate solution and centrifuged. The trypanosomes remained in the supernatant fluid, which was stored overnight in an icebox. It was then placed in a bath at from 40° C. to 50° C. for 20 minutes, which killed the trypanosomes. The prepared trypanosome containing plasma thus was injected into mice on 8 consecutive days, 24 hours after tumor inoculation, in doses of 0.25 cc., 0.35 cc., and 0.50 cc. On storage the serum lost its potency.

These experiments were made in order to determine whether the endotoxin specifically affected cancer cells, without producing any effect upon other organs and

tissues of the mouse. Seventy cancer-bearing mice were divided into 3 groups. The first group, of 43, was treated with trypanosome plasma. Ten mice, comprising the second group, were injected with plasma from healthy mice, prepared in the same manner. Seventeen cancer-bearing mice were untreated. The size of the tumors was recorded as follows: (a) scarcely palpable; (b) diameter up to 0.5 cm.; (c) 0.5 to 1 cm. in diameter; (d) 1 to 1.5 cm. in diameter; (e) very large.

On the 30th day the tumors in animals treated with prepared plasma measured on the average 1.01 cm., whereas those of control mice had a mean size of 3.23 cm. In all control animals the tumors developed well, whereas 19 of 43 mice injected with trypanosome plasma showed no tumors and 24 showed a prolonged latent period and slow growth. The 10 mice injected with normal mouse plasma and the 17 untreated controls developed the usual tumors.

Microscopic sections were made from tumors treated with the *Schizotrypanum cruzi* endotoxin. On the third day of treatment there appeared slight lymphocytic infiltration around the growth and small, scattered areas of necrotic cancer cells. On the fifth day these changes were more pronounced; numerous cancer cells were in various stages of necrosis, but among them healthy cells were present. A regressing tumor on the seventh day of treatment showed massive necrosis with replacement by spindle-shaped fibroblasts, while the intercellular spaces were filled with connective tissue fibers and lymphocytes. Ten days after the beginning of treatment an occasional cancer cell was noted, surrounded by granulation tissue.

Further experiments consisted in studying the therapeutic action of *Schizotrypanum* endotoxin obtained from trypanosomes cultivated *in vitro*. The culture was prepared in the same way as the *Schizotrypanum* plasma.

On the second day after tumor inoculation test animals received 0.3 cc. of endotoxin injected subcutaneously for 8 successive days. The mean index of tumor growth for experimental mice was 1.73 and for controls 4.52; tumors from 1 series weighed an average of 0.5 gm. for 12 treated animals and 3.39 gm. for 12 controls. In 10 of 23 treated mice complete recovery occurred.

The Action of *Schizotrypanum* Endotoxin *in Vitro*

A mouse tumor was reduced to pin-head fragments, and each one was placed in a drop of plasma containing *Schizotrypanum* endotoxin at room temperature (14° to 15° C.) for 6 hours and then implanted subcutaneously into 18 mice in the usual manner. Small fragments of the same tumor were immersed in normal mouse plasma at the same temperature and for the same time, and then grafted into 20 mice. Of the mice inoculated with grafts exposed to endotoxin, only 3 showed scarcely perceptible tumors on the 30th day, whereas those grafted with tumor kept in normal plasma developed tumors on the eighth to tenth day. On the 15th day tumors had grown in 10 of the 20; on the 30th day 6 mice died of their tumors, while the survivors developed large growths. The experiment clearly demonstrated a direct inhibiting effect of endotoxin on the tumor cell. Small tumor fragments kept in normal plasma for 24 hours after preliminary exposure to endotoxin plasma did not grow.

The effect of the reticuloendothelial system was studied by a preliminary administration of endotoxin serum and subsequent tumor inoculation. The reticuloendothelial system was blocked by *ferrum saccharum* in 15 mice, and by trypan blue in 15 additional ones. Twenty-two mice were splenectomized and then inoculated with tumor. Injections of *Schizotrypanum* endotoxin inhibited the growth of mouse cancer by its direct effect upon cancer cells and by stimulating the reticuloendothelial system. When the latter was blocked the therapeutic effect was absent.

The Action of *Schizotrypanum* Endotoxin on Human Tumors

Treatment was carried out by Dr. Bonhard in 3 patients with incurable cancer of the pharynx, chosen because changes could be easily observed.

Endotoxin was prepared in the following way: 4 cc. of heart's blood was taken at the height of infection from a guinea pig inoculated with *Schizotrypanum cruzi*, 1.5 cc. of a 2 per cent citrate solution was added, and the mixture was slowly centrifuged for 30 minutes. The plasma was drawn off and placed in an icebox (1° to 2°) for 20 hours, then inactivated at 58° C. for 30 minutes, and 1 cc. of a 1:1,000 rivanol solution was added to 10 cc. of plasma after it had cooled. Finally, the plasma was tested for contamination and stored in 1 cc. ampules. It lost its potency in about 10 days.

Injections were made into the tumor with a special syringe fitted with a platinum needle; the first dose was 0.5 cc., the second 1 cc., and the final one 2.0 cc. The treatment was repeated every other day.

One case history is given below.

K., male, aged 42, complained of severe dysphagia. Bilateral cervical adenopathy present. The pharynx showed a large bleeding tumor and biopsy verified the diagnosis of a malignant growth. The Oncologic Institute did not consider the patient a good risk for x-ray therapy. After 2 months' treatment with 16 endotoxin injections the nodes diminished in size, pain ceased, the tumor shrank, bleeding had stopped, and

the arytenoid cartilages showed mobility. The patient, who had gained 2 kgm. in weight, was then sent again for x-ray treatment. Three months later the pharynx showed considerable improvement. The general condition was good and the patient remained under further observation for about 2 years. In this one case treatment with the plasma reduced pain, prepared the case for x-ray treatment, alleviated inflammation, and stopped hemorrhage.

Our tentative trials suggest this form of therapy on more extensive material.

The Action of Bacterial Toxins

The effect of other bacterial toxins upon experimental cancer was then studied. Diphtheria and tetanus toxins, dysentery anatoxin, and *B. oedematiens* and *B. tumefaciens* toxins were tested.

The first two toxins gave positive results. The dysentery anatoxin and the *B. oedematiens* and *B. tumefaciens* toxins did not influence the development of tumors. In addition we carried out a series of experiments with preparations of nonpathogenic bacteria of the coli type; all failed to affect the tumor growth.

After a series of preliminary experiments we adopted the following procedure. Tumors were grafted in 1,000 animals and at the same time subcutaneous injections of toxin in various dilutions were given for several days. The size of the growth was recorded and at the end of the experiment all the tumors were excised, weighed, and their weights compared with those of tumors from control animals. Tetanus toxin caused regression in 19 of 48 treated mice, while 6 showed definite inhibition of tumor growth. The average weight of receding tumors was one-fifth that of controls. The effect of the toxin treatment was enhanced by ultraviolet irradiation of the experimental mice.

Diphtheria toxin gave better results. The amount and concentration of the injected diphtheria toxin (M.L.D. 0.002) is important in treatment, and was established after a number of tests. In definite doses this toxin not only inhibited tumor growth but also caused recession in a considerable number of animals. This effect appeared to a lesser degree in 10 day old tumors; their average weight in experimental animals was 1.63 gm. and in control mice 3.63 gm. Complete recovery occurred in 39 of 65 mice, while in 19 animals tumor growth was inhibited. The mean tumor weight was one-tenth that of control, untreated ones. The combined use of 1:400 toxin and ultraviolet radiation gave better results.

SUMMARY

1. Cancer cells may be particularly sensitive to certain protozoan endotoxins and bacterial toxins, while normal cells of a given animal species are immune.

2. Some bacterial toxins and protozoan endotoxins in adequate dosages inhibit the development of certain experimental tumors and cause complete regression of others.

3. Toxin therapy may become one of the methods for treating malignant tumors.

The Carcinogenicity of Wood Soot from the Chimney of a Smoked Sausage Factory

Edith Sulman, M.Sc., and Felix Sulman, M.D., V.M.D.

(From the Hormone Research Laboratory of the Hebrew University, Jerusalem, Palestine)

(Received for publication February 1, 1946)

In the course of a study of the estrogenic activity of plant materials in this laboratory (21) our attention was drawn to the fact that wood soot extracts have a slight estrogenic activity. It seemed of interest to ascertain whether this estrogenic activity is combined with a carcinogenic action.

In 1922 Passey (4) reported that extracts of soot collected from chimneys in England can evoke skin warts in animals. The well-known occurrence of scrotal carcinoma in chimney-sweeps, first described by Percivall Pott (1775), has been associated with the formation of carcinogenic substances in burning coal. Brownlie (1) has suggested that organic products of the decomposition of carbonaceous materials under high temperature conditions (especially bituminous coal, petroleum oil, coal-tar pitch, briquettes, illuminating gas, smoked foods, shale oils, etc.) can act as carcinogens. In numerous papers Roffo (5-17) has asserted that sometimes carcinogenic substances are formed by the burning at high temperatures of tobacco and coffee. Widmark (20) produced mammary adenocarcinomas in 40 per cent of female mice by applying extracts of roasted (275° C.) horse muscle, coffee, and brown butter. The spontaneous frequency of malignant tumors among his females was, however, rather high (10 per cent). Liu and Hu (3) produced papillomas in 4 out of 230 mice with tar obtained by destructive distillation of various vegetable foodstuffs. Dickens and Weil-Malherbe (2) produced 1 experimental skin papilloma and 8 pulmonary adenomas by means of wood smoke in 17 mice. Rats fed with smoked fish and meat did not, however, develop tumors during 20 months of observation. The experiments of Steiner, Steele, and Koch (18) suggest the possible carcinogenicity of overcooked meat, heated cholesterol, acrolein, and heated sesame oil. Whereas heating up to 300° C. did not produce carcinogenic substances, sesame oil that had been heated to 350° C. was carcinogenic in 3 out of 9 mice.

In view of these findings we undertook to ascertain:

(a) whether soot from wood is carcinogenic in our rat and mouse strains, which normally have a natural tumor incidence of about 0.1 per cent;

(b) whether the daily consumption of unlimited quantities of smoked sausage is carcinogenic for animals.

TECHNIC

The soot was obtained from the smoking chamber of a sausage factory in which eucalyptus timber was used for the smoking, where it had formed thick, black, coal-like masses. It was used either as fragments for implantation, or extracted from pulverized material with ether and afterwards with alcohol in a Soxhlet, and then injected. The extracts were pooled, the ether was evaporated at 40° C., and the alcoholic remnant was diluted with 96 per cent alcohol to a concentration that was one-tenth of the lethal dose for mice when 1 drop of the solution was licked. This precaution was taken in order to avoid primary mortality in mice painted with the extract, and secondary mortality in those sharing the same cage, for it is well known that mice sometimes lick extracts applied to the skins of their cage mates. Since separation of the mice used for this experiment was not advisable, care was taken to rub the soot extract well into the skin; thus optimal resorption ensued and little residue remained on the skin to be licked.

First Series

Thirty-six adult rats in all were implanted with pieces of soot weighing 5 to 20 mgm. in 18 (females, weighing 120 gm.) the particles were implanted subcutaneously near the right axilla; in the other 18 (males, weighing 150 gm.) the implantation was made in the scrotal sac between the testicles. In the male group tumor formation did not occur in the course of $2\frac{1}{2}$ years, a result that may have been due to low responsiveness of the site of implantation. Of the 18 females that had been implanted with the soot subcutaneously 3 reacted with sarcoma formation at the site of implantation after 12, 17, and 24 months respectively. These sarcomas did not metastasize in the original animals. When transplanted into 30 rats a take of about 50 per cent was recorded. In 36 male and female controls observed during the same interval no tumor was found.

Second Series

Ten adult female mice were treated with an alcoholic extract of soot on the dorsal skin of the neck once daily for 2 years. All in this group developed a cataract within a quarter of a year, a finding that sug-

gests the presence of derivatives of the naphthalene group. All the animals, furthermore, showed permanent estrus, which would indicate that the extract contained an estrogenic phenol derivative. The latter finding is hardly astonishing. Neoplasms occurred in 3 of the animals (30 per cent) and they died of their tumors after 5, 12, and 21 months respectively. Two of the tumors were sarcomas, situated in the vicinity of the urinary bladder, and one was a carcinoma of this viscus itself. No metastases were found. Implantations were made from each of the 3 tumors intraperitoneally near the kidney in 20 mice; only 1 take was recorded, this from a carcinoma implant. No neoplasms developed in 20 control mice of the same strain during an observation period of 2 years.

Third Series

Twenty adult rats were fed for 2 years on a diet that contained smoked sausage *ad libitum*. None of this group reacted with tumor formation during the period of the experiment.

DISCUSSION

The consumption of smoked sausage over a period of more than 2 years did not cause tumors in rats. However, the wood smoke used for its preparation was shown to contain substances that were carcinogenic for rats when introduced subcutaneously, and for mice when rubbed into the skin. The sarcomas obtained in 16.6 per cent of the rats following the implantation of soot particles may not appear significant, in view of the finding by Turner (19) that sterile bakelite disks, when similarly implanted in rats, elicited sarcomas in 31 per cent of the animals. Our findings become significant, however, by reason of the carcinogenicity of the soot extracts for mice (33.3 per cent) when rubbed into the skin. A further study of the possible carcinogenic role of wood soot extracts therefore seems desirable.

SUMMARY

1. Thirty-six female rats implanted subcutaneously with fragments of soot from the chimney of a sausage factory developed sarcoma in 16.6 per cent of the cases. No tumor developed in 36 male rats implanted intrascrotally with bits of the same soot.

2. Ten female mice treated for 2 years with an ether and alcohol extract of wood soot showed tumor formation in 3 cases (2 sarcomas and 1 carcinoma).

3. Twenty rats fed for 2 years on a diet containing an unlimited amount of smoked sausage failed to develop tumors.

4. The conflicting finding, carcinogenic effect in parenteral treatment versus absence of carcinogenic effect after oral administration, indicates the need for further study of the carcinogenic activity of smoked food, in view of the practical importance of the problem for human nutrition.

ACKNOWLEDGMENTS

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REFERENCES

1. Brownlie, D. The Cause of Cancer. London: Chapman and Hall, Ltd. 1938, pp. 8, 9, 32, 56, 67, 69, 182, 191.
2. Dickens, F., and Weil-Malherbe, H. Investigation into the Possible Carcinogenic Activity of Wood Smoke. Cancer Research, **2**:680-684. 1942.
3. Liu, Y., and Hu, C. H. Experimental Production of Tumors by Tars from Various Foods. Proc. Soc. Exper. Biol. & Med., **48**:226-227. 1942.
4. Passey, R. D. Experimental Soot Cancer. Brit. M. J., **9**: 1112-1113. 1922.
5. Roffo, A. H. El tabaco como cancerigeno. Bol. Inst. Med. Exp. & Cancer, Buenos Aires, **13**:287-336. 1936.
6. Roffo, A. H. El tabaco rubio como cancerigeno. Bol. Inst. Med. Exp. & Cancer, Buenos Aires, **15**:1-22. 1938.
7. Roffo, A. H. Unidad cancerigena de los alquitránes de diversos tipos de tabaco. Bol. Inst. Med. Exper. & Cancer, Buenos Aires, **15**:349-406. 1938.
8. Roffo, A. H. El alquitrán de café cancerigeno. Bol. Inst. Med. Exp. & Cancer, Buenos Aires, **15**:741-762. 1938.
9. Roffo, A. H. 1,2-Benzopirene, cancerigeno extraido del alquitrán del tabaco. Bol. Inst. Med. Exp. & Cancer, Buenos Aires, **16**:1-38. 1939.
10. Roffo, A. H. Tumours malignes développées dans l'appareil digestif par l'ingestion de graisses oxydées par chauffage. Bull. Assoc. franc. p. l'étude du cancer, **28**: 556-588. 1939.
11. Roffo, A. H. Sobre los filtros en el tabaquismo. El narguilé, y el algodon como filtro del alquitrán de tabaco. Bol. Inst. Med. Exp. & Cancer, Buenos Aires, **16**:255-268. 1939.
12. Roffo, A. H. Alquitrán de café cancerigeno. Bol. Inst. Med. Exp. & Cancer, Buenos Aires, **16**:445-446. 1939.
13. Roffo, A. H. Cancerizacion gástrica experimental por ingestion de grasses oxidadas. Bol. Inst. Med. Exp. & Cancer, Buenos Aires, **19**:503-530. 1942.
14. Roffo, A. H. Carcinoma tabáquico de gran malignidad desarrollado en un conejo. Bol. Inst. Med. Exp. & Cancer, Buenos Aires, **20**:1-50. 1943.
15. Roffo, A. H. Carcinoma del pulmón experimental par alquitrán tabáquico. Bol. Inst. Med. Exp. & Cancer, Buenos Aires, **20**:103-122. 1943.
16. Roffo, A. H. Sobre la malignidad de los tumores producidos par alquitrán tabáquico. Bol. Inst. Med. Exp. & Cancer, Buenos Aires, **20**:189-202. 1943.
17. Roffo, A. H. Cancerización gástrica por el alquitrán de café. Bol. Inst. Med. Exp. & Cancer, Buenos Aires, **20**: 425-470. 1943.
18. Steiner, P. E., Steele, R., and Koch, F. C. The Possible Carcinogenicity of Overcooked Meats, Heated Cholesterol, Acrolein, and Heated Sesame Oil. Cancer Research, **3**: 100-107. 1943.
19. Turner, F. C. Sarcomas at Sites of Subcutaneously Implanted Bakelite Disks in Rats. J. Nat. Cancer Inst., **2**: 81-83. 1941.
20. Windmark, E. M. P. Presence of Cancer-Producing Substances in Roasted Food. Nature, London, **143**:984. 1939.
21. Zondek, B., and Bergman, E. Phenol Methyl Ethers as Oestrogenic Agents. Biochem. J., **32**:641-645. 1938.

Influence of Crown-Gall Bacterial Products, Crown-Gall Tissue Extracts, and Yeast Extract on Growth *in Vitro* of Excised Tobacco and Sunflower Tissue*

Albert C. Hildebrandt, Ph.D., A. J. Riker, Ph.D., and B. M. Duggar, Ph.D.**

(*From the Department of Plant Pathology, University of Wisconsin, Madison 6, Wisconsin*)

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The influence on the growth of plant tissue *in vitro* of various extracts from crown-gall bacteria, yeast, and crown-gall tissue has been studied with the hope of clarifying their effect on the metabolism of abnormally proliferating cells. This has appeared to be one important means of examining the basic vital processes involved in the initiation and continuation of pathological growth.

The value of plant materials as tools for studying tissue metabolism is seen (24), *e.g.,* in their large experimental numbers, low cost, ease of experimental use, physiological variability, easily induced epidemics, genetic purity, and cultivation of isolated tissue on a medium containing only nutrients for which chemical formulas are known.

The crown-gall disease of plants, incited by *Phytomonas tumefaciens,* and related non-parasitic proliferations offer excellent technical opportunities because of certain fundamental similarities betweeen these diseases in plants and cancer in animals. Considerable information (10, 25) is available on the anatomy of the crown-gall tissue and the physiology of the causal organism.

This microorganism is studied, not because of a particular interest in parasitism *per se,* but rather because in tissue this bacterium produces metabolites in low concentrations over a long period of time. This is, in a way, comparable to "irritation" from relatively insoluble carcinogenic agents. However, these bacterial metabolites are in a different class of chemicals from the known carcinogens and, therefore, provide a different approach to the basic problem. While these bacteria have been thoroughly studied, little is known about the physiology of the host cells.

The culture of isolated plant tissue on a completely synthetic medium *in vitro* offers various advantages for study. For example, the influences of other and different tissues are eliminated, and thus the cells grow or fail to grow depending (a) on what is present in the culture, either originally or under the influence of tissue metabolism; or (b) on what is absent from it. This enlarges considerably the scope of investigation on the fundamental metabolism of higher plant cells.

The possibilities and advantages of a plant tissue culture technic in fundamental physiological research ocurred long ago to Haberlandt (5), who kept a variety of cell types alive *in vitro* for several weeks. By 1922 Robbins (27) showed that isolated corn-root tips *in vitro* would live through several passages, and he had many essentials of the problem worked out. In 1934 White (31) grew tomato roots through an indefinite number of passages. Following the methods developed for culturing isolated root tips, White (32) with tobacco tissue, as well as Gautheret (3) and Nobecourt (21), both with carrot tissue, successfully developed true plant tissue cultures. The extensive literature has been reviewed by White (33).

The best conditions for callus [1] growth *in vitro* are still obscure, although much is known about root-tip culture. Therefore the influence of temperature, acidity, and sucrose concentration was studied first (8). These data provided a necessary foundation for clarifying studies of the factors involved when the metabolites of crown-gall bacteria and other substances were examined.

The products of crown-gall bacteria have been studied extensively, and the work has been reviewed by Riker and Berge (25) and by Riker (24). However, neither these substances nor extracts from crown-

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[1] Haberlandt (5) hoped to culture individual, isolated cells. This has not yet been accomplished. In the present paper it seems advisable to consider all cultures of masses of isolated cells under the broad heading of callus or tissue cultures.

gall and healthy plant tissues have been employed against callus growth *in vitro*.

The present paper (7), which has appeared in abstract, reports the influence of supplements of fermented media from crown-gall bacterial cultures, and of crown-gall tissue extracts, on the growth *in vitro* of excised sunflower and tobacco callus cultures. In the study of diseased growth it seems important to know whether extracts of these materials *in vitro* can either stimulate or inhibit the proliferation of this callus, which is so closely related to, if not identical with, atypical and pathological tissue.

METHODS

Tissues from two plant species were used in these studies. The tobacco tissue was from the hybrid *Nicotiana glauca* Grah. ♀ × *N. langsdorffii* Weinm. ♂. It was isolated originally and was supplied by Dr. P. R. White. The other tissue was isolated from "secondary" petiolar crown gall on sunflower (*Helianthus annuus* L., var. Giant Russian) and was free of the bacteria (8). Both the tobacco and sunflower tissues are capable of unlimited growth *in vitro*.

Crown-gall bacteria were present in a certain percentage of the originally isolated gall tissues, and they produced a heavy growth over the surface of both the medium and the tissue. In one set of isolations from 50 "secondary" galls, 16 of the isolates contained crown-gall bacteria. Such cultures were discarded. The basic medium was excellent for the growth of the bacteria, which have not appeared, as reported by White and Braun (34), from the isolated tissues carried beyond the first subculture. Crown-gall bacteria were never observed in the stock cultures of sunflower tissue, which were derived from one "secondary" crown gall. Many successive subdivisions, made at monthly intervals since December, 1941, have continued vigorous growth.

Stock cultures of sunflower and tobacco tissue were kept in diffuse light at room temperature. The basic medium (33) as used in these studies contained the following quantities of salts in milligrams per liter of distilled water: 360 $MgSO_4 \cdot 7H_2O$, 200 $Ca(NO_3)_2 \cdot 4H_2O$, 200 Na_2SO_4, 80 KNO_3, 65 KCl, 16.5 $NaH_2PO_4 \cdot H_2O$, 2.5 $Fe_2(SO_4)_3 \cdot 6H_2O$, 4.5 $MnSO_4 \cdot 4H_2O$, 1.5 $ZnSO_4 \cdot 7H_2O$, 1.5 H_3BO_3, and 0.75 KI. In addition the medium contained 20 gm. sucrose, 7.5 gm. agar, 3.0 mgm. glycine, and 0.1 mgm. thiamine hydrochloride per liter. All chemicals were reagent grade except the sucrose, boric acid, and potassium iodide.

A record was kept of the source and impurities of the ingredients, but it is omitted here to conserve space. The agar was leached by washing three times daily in distilled water for several days. The agar was autoclaved several times in the process of making a concentrated stock water-agar. This and the leaching destroyed some of its gel-forming properties, so that when the medium was finally made it had more the thickness of a 0.5 per cent agar medium. The basic medium was prepared from concentrated stock solutions as described by White (33). All experiments were made on 50 ml. of medium in 125 ml. Erlenmeyer Pyrex flasks, which had been through cleaning solution and had been rinsed in tap and distilled water.

The experimental cultures were started by placing stock tissue in a sterile Petri dish; after it had been cut with a sterile scalpel into irregular, hexahedral pieces 3 mm. in greatest dimension and weighing 20 to 30 mgm., 4 good pieces were transferred to each flask. When various treatments were studied the seed pieces from each stock tissue piece were distributed equally as far as possible among the different treatments. All tissue transfers were carried out aseptically in a special transfer room. The experimental cultures were incubated in the dark at $26° \pm 1°$ C. Six flasks, each containing 4 tissue pieces, were used per treatment, and each experiment was repeated at least 3 times unless otherwise noted. The tissues were removed from the culture flasks after 6 weeks' incubation, and the amount of growth was determined by weighing each piece individually to the nearest 0.01 gm.

Bacterial culture media of the virulent and attenuated strains of the crown-gall organism, which were used as supplements with the tissue cultures, were obtained as follows: The bacteria were incubated (for the medium and shaking method see McIntire, Peterson, and Riker) for 5 days at $26° \pm 1°$ C. in shake cultures, were largely removed with a Sharples centrifuge, and were more completely removed with a Seitz filter. To make doubly certain of sterility the medium was filtered through suitable fritted-glass filters and tested on culture media. No bacterial contamination appeared during a 6 weeks' incubation of the tissue.

Crown-gall tissue extracts were prepared from 6 week old galls. The galls were cut from marigold and tomato plants grown in the greenhouse and from Paris daisy plants grown in the field, frozen for 24 hours with dry ice, ground in a food chopper, and then thawed. The sap was extracted by 8,000 lb. pressure per sq. inch. The larger particles were removed from the extracted sap by centrifuging and by filtering through a Seitz filter, and the filtrate was sterilized by autoclaving or by fritted-glass filters.

RESULTS

Variability of the tissue.—The wet weight of the tissue after 6 weeks' incubation indicated the measure

of growth. The data from all experiments, which were repeated several times during the year, were analyzed by the analysis of variance.[2] For any individual run 6 flasks containing 24 tissue pieces were usually employed for each treatment (Table I). In the analysis of the combined data from all runs of a given extract (Table II) the variability within treatments was separated into two parts (Table III); *i.e.*, the variability between different runs within a treatment and the variability between different flasks within a run within a treatment. The first of these two sources of variability is the best estimate of error to compare the differences between treatments when it is desired to answer the question: Are the differences found likely to be obtained if the experiment is repeated? The second source of error is

TABLE I: TOTAL WET WEIGHT OF SUNFLOWER TISSUE CULTURES GROWN ON THE BASIC MEDIUM SUPPLEMENTED WITH VARIOUS CONCENTRATIONS OF MARIGOLD CROWN-GALL TISSUE EXTRACT

Extract concentration *	"Replicates" 1	2	3	4	5	6	Average
0	0.68	0.74	0.54	0.93	1.04	0.92	0.81
1/8	1.24	1.31	1.43	1.78	1.25	1.73	1.86
1/4	1.98	1.50	1.74	1.51	2.86	2.27	1.98
1/2	2.23	1.99	2.70	2.84	3.10	3.13	2.67
1	2.90	2.25	3.10	3.67	3.32	2.94	3.03
2	3.87	2.67	4.15	4.00	4.11	3.41	3.71
4	2.21	3.30	4.60	5.20	4.56	5.04	4.15
8	0.58	2.96	2.77	0.59	3.09	4.28	2.38

* Concentration of gall-extract supplements in ml. per 50 ml. of medium.

Least significant difference for between-concentration averages equals 0.89 and 1.19 gm. respectively, at the 5 and 1 per cent levels.

TABLE II: TOTAL WET WEIGHT OF TOBACCO TISSUE CULTURES GROWN ON THE BASIC MEDIUM SUPPLEMENTED WITH VARIOUS CONCENTRATIONS OF AUTOCLAVED MARIGOLD CROWN-GALL TISSUE EXTRACT

Extract concentration *	"Replicates" † 1	2	3	Average per flask ‡
0	4.85	5.92	4.76	0.86
1/8	8.74	7.13	6.68	1.25
1/4	11.86	8.96	8.60	1.63
1/2	16.01	7.86	8.29	1.79
1	17.88	13.42	8.57	2.22
2	22.21	17.12	9.64	2.72
4	24.52	10.53	8.26	2.40
8	14.64	0.66	4.25	1.08

* Concentration of gall-extract supplements in ml. per 50 ml. of medium.

† Each number represents the total wet weight in grams of 24 tissue pieces from experiments run (a) June 23, 1943, to August 3, 1943; (b) December 22, 1943, to February 2, 1944; and (c) December 23, 1943, to February 4, 1944.

‡ Average wet weight in grams of 4 tissue pieces per flask. Least significant difference for the average weights at the 5 and 1 per cent levels was 0.95 and 1.26 gm., respectively.

[2] The authors are grateful to Dr. J. H. Torrie and Virginia B. Beal for their assistance in this phase of the problem.

TABLE III: SUMMARY OF ANALYSES OF VARIANCE FOR BETWEEN-TREATMENTS AND FOR BETWEEN-TREATMENTS RUN AT DIFFERENT TIMES WITH CROWN-GALL TISSUE EXTRACTS, YEAST EXTRACT, AND BACTERIAL CULTURE MEDIA SUPPLEMENTS AS DETERMINED FROM THE COMBINED RESULTS OF SIMILAR EXPERIMENTS MADE AT DIFFERENT TIMES

	Extract Supplement: Tomato M.S.*	F †	Marigold M.S.	F	Paris daisy M.S.	F	Yeast M.S.	F	Medium of: Virulent culture M.S.	F	Attenuated culture M.S.	F
Sunflower tissue cultures:												
Treatments	2.90	17.25 § (8.25 §)	7.93	3.52 ‡ (9.82 §)	4.13	2.41 (12.13 §)	4.62	6.08 § (22.05 §)	0.55	5.90 § (18.27 §)	0.50	4.86 ‡ (7.69 §)
Times	4.32	25.64 § (12.29 §)	22.76	10.60 § (28.19 §)	10.83	6.19 ‡ (31.13 §)	15.89	20.95 § (75.93 §)	1.61	17.34 § (53.70 §)	0.49	4.78 (7.57 §)
Treatment × Times	0.17		2.14		1.75		0.76		0.09		0.10	
Error	0.35		0.81		0.35		0.21		0.03		0.07	
Tobbaco tissue cultures:												
Treatments	0.92	32.68 § (62.91 §)	0.08	1.01 (4.79 §)	0.28	57.35 § (58.57 §)	0.06	6.26 § (2.37 ‡)	0.04	1.26 (2.68 ‡)	0.04	1.52 (3.35 §)
Times	0.33	11.75 § (22.62 §)	1.15	19.01 § (71.95 §)	0.10	20.90 § (21.34 §)	0.33	37.27 § (14.12 §)	0.57	19.13 § (40.72 §)	0.68	24.12 § (53.33 §)
Treatment × Times	0.03		0.08		0.05		0.01		0.03		0.03	
Error	0.02		0.02		0.05		0.02		0.01		0.01	

* Mean square.

† (Upper figure) F value calculated using treatment × time mean square. (In parentheses) F value calculated using error mean square.

‡ F value significant at 5 per cent level.

§ F value significant at 1 per cent level.

applicable only to compare differences within an individual run, and in no way takes into account the variability from run to run.

The data from one representative experiment (Table I) are from a single trial on the effect on growth *in vitro* of excised sunflower tissue by various concentrations of an autoclaved extract from crown-gall tissue of marigold. Each figure under "replicates" 1, 2, 3, 4, 5, and 6 represents the total wet weight in grams of 4 tissue pieces in a single flask. There were 7 different concentrations of the extract, plus the control with no extract supplement, or 8 treatments. The analysis of variance for the data in Table I gave a highly significant F value (12.73) for between-treatments that was several times above the 1 per cent level (3.12).

To verify results from individual experiments, statistical analyses were used on the combined results of similar experiments repeated at different times. The total weight in grams of 24 tissue pieces in the 6 flasks with the same treatment from 1 experiment was considered as 1 replicate. A representative example of the data for such an analysis of variance for the effect of autoclaved marigold crown-gall tissue extract on growth *in vitro* of sunflower tissue is presented in Table II. Differences between different concentrations of marigold crown-gall tissue extract (Table II) were significant. Differences between runs were also significant. Control cultures on the basal medium were made with each individual experiment.

Fermented bacterial cultures.—The effects of fermented culture media of *P. tumefaciens* on the growth of tissue cultures were examined with media from which, after 5 days, the cells of an attenuated (A6-6 strain) *P. tumefaciens* had been removed as described earlier. Similar studies were made with media of a sister but virulent culture (A6 strain). The fermented media, after sterilization by filtration, were added aseptically to the basic tissue culture medium before it solidified, in concentrations of 0, ⅛, ¼, ½, 1, 2, 4, and 8 ml. per 50 ml. respectively. No attempt was made to buffer the media. The influence of these supplements on the growth of excised sunflower and tobacco tissue *in vitro* is shown in Fig. 1. Each point on the curves for sunflower represents the average wet weight of 36 (2 trials) and 84 (4 trials) tissue pieces respectively for the media from attenuated and virulent strains; on the curves for tobacco tissue each point in the same order is the average wet weight of 48 (3 trials) and 60 (3 trials) tissue pieces respectively.

With sunflower tissue the supplements of fermented medium from the attenuated bacterial culture retarded growth at all concentrations except the most dilute. The influence of fermented medium from virulent cultures on the growth of sunflower tissue was similar to that with the attenuated medium, except that all concentrations were unfavorable for best growth.

Supplements of the attenuated and virulent bacterial culture media had only a slight effect on the growth of tobacco tissue cultures.

The data from experiments with each bacterial culture medium supplement were analyzed statistically. The F values for differences between treatments and for between times calculated from both the error, and treatment × times mean squares are summarized in Table III. The F values for between-treatments using the error mean square were highly significant in all cases except for the tobacco tissue and the supplement of the medium from virulent cultures. The F values for differences between treatments obtained by using the treatment × times mean square were significant with sunflower tissue, but not with tobacco tissue.

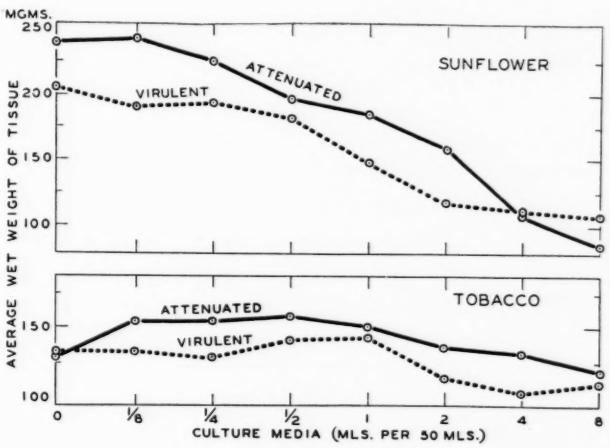

FIG. 1.—Effects of bacteria-free, fermented media of virulent and attenuated crown-gall cultures on excised tobacco and sunflower tissue growing *in vitro*.

Supplements of these media, therefore, seemed to have little effect at lower concentrations, but to have a retarding effect at the higher concentrations. With excised tomato roots Friedman and Francis (2) also observed lowered growth with an ether extract of crown-gall culture media.

The final pH of the media in these experiments indicated that their acidity increased with increasing concentrations of these supplements, and the final acidity of the fermented media ranged from pH 5.7 to 4.5. Thus, for sunflower tissue, when the attenuated culture medium was added at concentrations of ⅛ to 8 ml. per 50 ml. respectively, the final acidities averaged pH 5.7, 5.7, 5.7, 5.6, 5.5, 5.1, and 4.9, and with the medium from the virulent cultures and the same concentrations the average final acidities were

pH 5.5, 5.4, 5.5, 5.0, 5.0, 4.9, and 4.8. Media supplemented with the medium of attenuated cultures at concentrations from ⅛ to 8 ml. per 50 ml. on which the tobacco tissue was grown had average final acidities of pH 6.3, 6.2, 6.3, 6.1, 5.8, 5.1, and 5.1 respectively; and with the medium from virulent bacterial cultures at the same concentrations the average final acidities were pH 6.3, 6.1, 6.0, 6.0, 5.3, 4.9, and 4.8. The bacterial culture media contain ammonium sulfate (19), and the acidity of these fermented media may arise from utilization by the bacteria of part of the ammonium sulfate and the consequent formation of sulfuric acid. Reference to data on the effect of acidity on the growth of these tissues (8) suggested that the inhibiting effect of the culture-media supplements at the higher concentrations might result, in large part, from this increased acidity. However, there may be other materials produced by the bacteria, which retard the tissues and which are considered below.

Effect of lyophilized cells.—Certain materials isolated from the crown-gall bacterial cells and considered important in this type of growth suggested investigations with lyophilized cells. For example, a fat, a phosphatide, and one or more polysaccharides have been isolated by different workers from the bacterial cells, and their influence on plant growth has been observed (13). The chemical composition of the cells has been studied by Anderson and his coworkers (see Velick). Geiger and Anderson (4) analyzed the lyophilized virulent cells from 2 media and found them to contain approximately the following: nitrogen, 5 to 10 per cent; phosphorus, 2 to 4 per cent; sulfur, 0.3 per cent; ash, 7 to 27 per cent; and moisture, 5 to 8 per cent. The bacteria on sucrose medium gave 6 per cent of total lipids, of which 64 per cent was phosphatide, the latter consisting of about equal parts of lecithin and cephalin. The influence of the whole lyophilized cells on growth of callus tissue is briefly described here.

The lyophilized cells, prepared as described by Locke, Riker, and Duggar (15), were sterilized by autoclaving for 20 minutes at 15 lb. pressure in concentrated water suspension and added aseptically to the basic medium before it had solidified, to give dry weight concentrations of 0, 0.003, 0.006, 0.012, 0.025, 0.05, 0.1 and 0.2 gm. per 50 ml. of the basic medium.

Supplements to the basic medium of lyophilized virulent cells were slightly beneficial at concentrations of 0.003 and 0.006 gm. per 50 ml., but detrimental at higher concentrations for sunflower tissue. In two experiments (36 tissue pieces at each concentration) a significant F value was found in only one. One run (12 tissue pieces at each concentration) with attenuated cells gave no significant difference between treatments.

The final acidity of cultures with the lyophilized virulent cells at concentrations from 0.0016 to 0.1 gm. per 50 ml. averaged, respectively, pH 5.8, 5.8, 5.7, 5.6, 5.6, 5.4, and 5.5; and with the attenuated cells at concentrations from 0.003 to 0.2 gm. per 50 ml., pH 5.9, 5.6, 5.5, 5.4, 5.3, 5.2, and 5.2 respectively. These acidities, except for the cultures with attenuated cell supplements at concentrations of 0.1 and 0.2 gm. per 50 ml., were within the range for best growth of this tissue.

With tobacco tissue there was a progressive decrease in growth with increase in concentration of the lyophilized bacterial cells. From a statistical standpoint, the results of two experiments (36 tissue pieces at each concentration) with cells of the virulent strain were highly significant.

The inhibiting effects of the bacterial cell supplements at higher concentrations were associated with an increased acidity. Thus the final acidity for cultures supplemented with lyophilized virulent cells at concentrations from 0.003 to 0.2 gm. per 50 ml. respectively averaged pH 6.1, 6.1, 5.8, 5.5, 4.8, 4.5, and 4.4; and with the attenuated cells at concentrations from 0.0016 to 0.1 gm. per 50 ml., pH 6.0, 5.8, 5.7, 5.4, 5.3, 4.9, and 4.7. The increased acidity at the higher concentrations would be unfavorable for best growth of the tissue.

Extracts of yeast and crown-gall tissue.—The influence of yeast extract and of crown-gall tissue extracts was of interest. Numerous reports with similar extracts on the growth of other excised tissues have appeared. For example, Robbins (27) and White (31) observed the beneficial action of autolyzed yeast extract on tissue cultures. Leaf extracts of a number of species stimulated the growth of excised root tips of the same and different species (16, 17), while extracts of corn grains proved beneficial to corn root cultures (28). Overbeek, Conklin, and Blakeslee (22) found unautoclaved coconut milk necessary to culture small embryos of *Datura,* but Blakeslee and Satina (1) reported recently that unautoclaved powdered malt extract would replace this "embryo factor." The growth substance content of gall-tissue is of interest because of the possible relationship of such substance to the stimulation of pathological growth.

The growth substance content of the gall-tissue has been investigated in part; in general, the proximate analyses resemble those of young plants (20). The composition of crown-gall tissue from tomato plants grown in the greenhouse is given by Nagy, Riker, and Peterson (20) on the basis of dry matter approximately as follows: ash, 13.2 per cent; total nitrogen, 3.3 per cent; carbohydrate, 34.2 per cent; and

uronic acids, 10.9 per cent. Locke, Riker, and Duggar (13) found more than the normal amount of growth substance, like heteroauxin, in tomato-gall tissue when comparisons were made on a total weight basis, but later Riker, Henry, and Duggar (26), in more critical studies, reported no significant difference in auxin content, when compared on a total nitrogen basis, between inoculated and control tissues of tomato 1 to 16 days after inoculation. Link and Eggers (12) found that extracts of gall-bearing hypocotyls of tomato gave higher free and potential auxin assays than extracts of normal healthy hypocotyls. They, and Nagy, Riker, and Peterson (20) also, found gall-tissue richer than contiguous tissue in ash, ether extracts, total nitrogen, and simple forms of nitrogen; glutathione and vitamin C were more abundant, and the activity of oxidizing enzymes was higher. Henry, Riker, and Duggar (6) also reported that galls on tomato consistently contained more thiamine than healthy tissue. With such a background, experiments were planned to determine the influence of crown-gall tissue extracts and of yeast extract on growth *in vitro* of sunflower and tobacco tissue.

The gall-extracts were prepared as previously described. The yeast extract was made by suspending 20 gm. of dried brewer's yeast in 200 ml. of distilled water, cooking this in a steamer for 1 hour, filtering it through asbestos, and bringing the filtrate up to a 200 ml. volume with distilled water. Paris daisy extract and marigold extract were sterilized by autoclaving, while yeast extract and tomato extract were sterilized by filtration through fritted-glass filters. These were added aseptically to the basic medium, before it solidified, in concentrations from 0 to 8 ml. per 50 ml. of medium. No special attempt was made to buffer the medium. The pH was determined at the end of the 6 week incubation period.

The average wet weight of sunflower and tobacco tissue after 6 weeks is shown in Fig. 2. Each point represents the average wet weight of 72 tissue pieces from experiments repeated at least 3 times, unless otherwise noted. The F values for between-treatments and for between-treatments in similar series run at different times, as determined by statistical analyses of the results, are summarized in Table III. The influence of each of the extracts is considered below.

Increased growth of sunflower tissue was obtained over controls with all concentrations of marigold extract employed with the best growth at 2 ml. per 50 ml. of medium. The average final acidity of the media at concentrations from 0 to 8 ml. per 50 ml. was pH 5.9, 5.9, 5.9, 5.8, 5.7, 5.6, 5.4, and 5.2 repectively. Tomato crown-gall tissue extract was beneficial for growth of sunflower tissue at all concentrations except at 8 ml. per 50 ml. Best growth appeared at concentrations of 1 and 2 ml. per 50 ml. of medium. The average final acidity at concentrations from 0 to 8 ml. per 50 ml. was pH 5.7, 5.8, 5.9, 5.8, 5.8, 5.8, 5.8, and 5.5 respectively. Yeast extract was favorable also for growth of sunflower tissue at all concentrations except 8 ml. per 50 ml. of basic medium. A concentration of ½ ml. per 50 ml. of medium resulted in best growth. Statistical analyses of the data from 3 experiments with a total of 76 tissue pieces at each concentration showed that differences between treatments were highly significant (Table III).

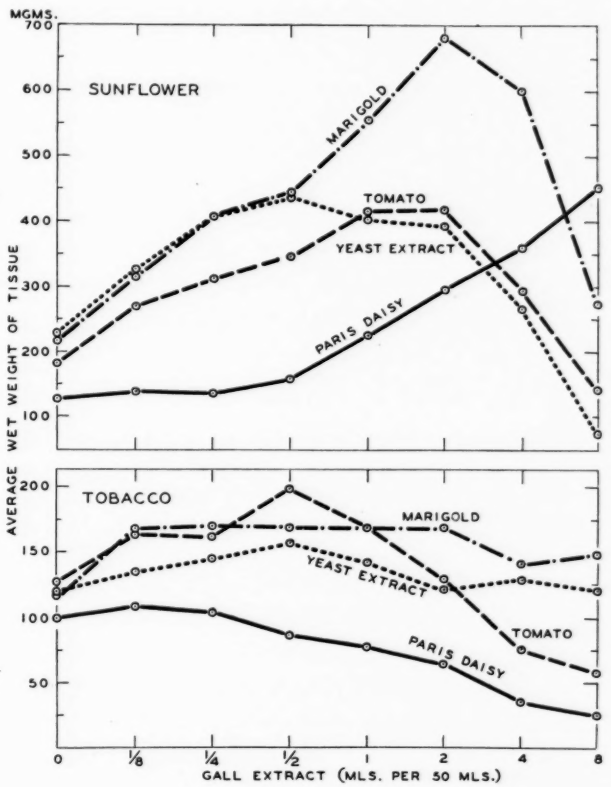

FIG. 2.—Effects of various extracts, respectively, from crown-gall tissue on several host plants and of 10 per cent autolysate of dried brewer's yeast on excised sunflower and tobacco tissues growing *in vitro*.

The average final acidity of media with concentrations of yeast extract from 0 to 8 ml. per 50 ml. was pH 5.3, 5.3, 5.3, 5.2, 5.1, 5.0, 5.1, and 5.3 respectively. Increased weight of sunflower tissue occurred with increased concentrations of Paris daisy-gall extract throughout the range employed. The average final acidity of the media at concentrations from 0 to 8 ml. per 50 ml. was pH 5.6, 5.6, 5.5, 5.6, 5.4, 5.4, 5.2, and 5.3 respectively. Differences between treatments were highly significant as determined from the results of 3 runs with a total of 72 tissue pieces at each concentration (Table III).

Marigold-gall extract favored growth of tobacco tissue at all concentrations. Concentrations from ⅛

to 2 ml. per 50 ml. of medium resulted in best growth. The average final acidities of the media at concentrations from 0 to 8 ml. per 50 ml. were pH 5.8, 6.0, 6.0, 5.9, 5.9, 5.7, 5.3, and 5.2 respectively. Analyses of 3 experiments with a total of 60 tissue pieces at each concentration gave a highly significant F value for between-treatments. Yeast extract had only a slightly beneficial effect on growth of tobacco tissue. The best concentration was ½ ml. per 50 ml. of medium. The average final acidity of media with concentrations from 0 to 8 ml. per 50 ml. was pH 5.9, 5.7, 5.8, 5.7, 5.3, 4.8, 4.7, and 4.7 respectively. An analysis of variance of data from 3 runs, totaling 76 tissue pieces at each concentration, showed significance between treatments at the 5 per cent level. Tomato-gall extract improved growth of tobacco tissue at concentrations from ⅛ to 1 ml. per 50 ml., but was harmful at from 4 to 8 ml. per 50 ml. of the basal medium. The greatest average wet weight of tissue was found on medium containing ½ ml. of the extract. The average final acidity of the media with concentrations from 0 to 8 ml. per 50 ml. was pH 5.9, 5.8, 5.9, 5.8, 5.7, 5.4, 5.3, and 5.4 respectively. A highly significant difference between treatments was found by analysis of the data from 4 experiments with 100 tissue pieces at each concentration. Paris daisy-gall extract had little effect on growth of tobacco tissue at low concentrations but was harmful at high concentrations. The average final acidity of the media with 0 to 8 ml. per 50 ml. was pH 5.8, 5.7, 5.8, 5.7, 5.5, 5.0, 4.5, and 4.6 respectively.

The significance of the results with different extract supplements is summarized in Table III. Differences between extract concentrations as determined from the error mean square were highly significant in each case except with yeast extract and tobacco tissue, where the differences were significant at the 5 per cent level. Differences between treatments as determined with the treatment × times mean square were significant in each case except when Paris daisy-gall extract was used in sunflower tissue cultures, and when marigold-gall extract was used with tobacco tissue cultures.

Table III also presents the F values for between-experiments (time) means. The differences between times were highly significant in each case except where Paris daisy-gall extract was used in connection with sunflower-tissue cultures.

The acidity of the medium in each culture was determined at the end of the incubation period. Thus its final acidity with Paris daisy extract supplements from 0 to 8 ml. per 50 ml. of medium on which the tobacco tissue was grown was pH 5.8, 5.7, 5.8, 5.7, 5.5, 5.0, 4.5, and 4.6 respectively. The inhibiting effect of the Paris daisy-gall extract on tobacco tissue at concentrations of 2, 4, and 8 ml. per 50 ml. can be explained partly by the correspondingly increased acidity at these concentrations. There was also an increased acidity with tobacco tissue at higher concentrations of yeast extract. The acidity with other extracts and both tissues was not unfavorable for growth of the tissues. The actual readings are given above. Although the decreased growth of tobacco tissue appearing with higher concentrations of yeast extract and Paris daisy-gall extract was associated with a slightly unfavorable acidity, there was also decreased growth of the tissue with the same and other extracts, even though the acidity was favorable for growth. This suggested that there were factors other than increased acidity active in inhibiting growth at the higher concentrations of the extracts.

The extracts generally were stimulating to both tissues at the lower concentrations, but inhibiting at the higher. The beneficial effect of factors in yeast extract for growth of excised roots is well known. The stimulating effect of the plant extracts usually was greater than that of the yeast extract. The results indicated the necessity for an understanding of the influence of each of the various factors introduced with these extracts. Supplements of tissue extracts introduce a variety of changes in the basic medium. For example, such supplements contain in differing concentrations various sources of carbon, nitrogen, and mineral salts; amino acids; vitamins; and other growth substances.

DISCUSSION

The effects of crown-gall bacterial metabolites and crown-gall tissue extracts on growth *in vitro* of excised tobacco and sunflower tissue are important for understanding the pathological growth incited by the bacteria.

Various known and probably certain unknown compounds were added to the basic medium when the fermented virulent and attenuated culture media were used as supplements. The bacterial culture medium contained, among other things, ammonium sulfate, urea, magnesium sulfate, zinc sulfate, and ferric alum at concentrations of 1.0, 0.5, 0.2, 0.002, and 0.005 gm. per liter respectively. The sucrose in the bacterial culture media was utilized largely by the bacteria during the 5 day culture period. Certain amounts of calcium and sodium were added as chlorides, and 1.0 gm. of phosphate was used per liter.

Various materials were added similarly when the basic medium was supplemented with the bacterial cells (4), with the yeast extract (9), and with the crown-gall tissue extracts (20).

In view of results obtained with improved salt concentrations (7) and with different sucrose concen-

trations (8), and in view of the chemical composition of these various supplements, it appeared that the beneficial effect was due in part to the increased and more favorable concentrations of the basic nutrient materials, or to the addition of other desirable nutrients not present or present in insufficient quantities in the basic medium. For example, increased concentrations of magnesium sulfate, iron, zinc, and calcium provided by the fermented media may be favorable for growth of the tissues. The same conditions may prevail with the other salts introduced by the fermented media, the amount introduced depending upon the amount previously utilized by the bacteria. The action of urea provided by the bacterial media may be beneficial to the tissues.

Favorable factors for the tissues may have been synthesized by the crown-gall bacteria from such compounds. Locke, Riker, and Duggar (14) showed an increased heteroauxin content in fermented virulent and attenuated culture media over control cultures. Unpublished data indicated that indole-acetic-acid produced by 5 day old crown-gall cultures in 8 ml. of medium would have little effect on tobacco tissue cultures, but might be toxic to the sunflower tissue. McIntire, Riker, and Peterson (18) also found that biotin, pantothenic acid, and riboflavin were synthesized by the bacteria. The high ash content of the lyophilized cells (4) also would suggest the introduction of inorganic salts by these supplements. Sucrose contributed by the fermented media was probably not important in the stimulation observed, because most of the sugar was utilized by the bacteria during the 5 day culture period.

The products, as used in these experiments, were lacking in the stimulating effect produced in plants by inoculation with the living bacteria. Possibly there should be more intimate contact between the bacterial cells and the host, as occurs in the inoculated plant, if there is to be the typical pathological response.

Inhibiting effects of these supplements were evident at the higher concentrations. Levine and Chargaff (11) reported similar inhibiting or toxic effects on intact plants by certain crown-gall bacterial products. The fact that the bacterial cells, culture media, and polysaccharide had little stimulating effect might indicate that this stimulating principle, if present in higher concentrations in the original preparations, either was destroyed at least in part in the process of preparation of these products, or was counteracted by the development or presence of more inhibiting or toxic factors at the higher concentrations. Most of the inhibiting or toxic effect on tobacco tissue observed with higher concentrations of fermented media may be due to the increased acidity of around pH 5.0 to 4.8. The utilization of ammonia in the ammonium sulfate by the bacteria may be responsible for the change in hydrogen-ion concentration of the bacterial culture medium. However, there doubtless are other factors responsible for inhibition at the higher concentrations.

Crown-gall tissue extracts and yeast extract contained some factor or factors favorable to growth of excised tobacco and sunflower tissue (Fig. 2). This stimulating action may be due to one or more factors. Deficiency of the basic medium in organic and inorganic nutrients immediately suggests itself as a reason for the beneficial action of these extracts. Yeast is known to be rich in growth factors, and yeast and gall tissues are also high in ash, carbohydrate, and protein. Just (9) gives the composition of brewer's yeast approximately as follows: total nitrogen, 7.2 per cent; protein, 43 per cent; fat, 2 per cent; carbohydrate, 49 per cent; and ash, 6.9 per cent.

The data also indicate that not only stimulating but inhibiting or toxic factors as well were present in the various extracts. Similar inhibiting effects with other extracts have been reported; for example, by Robbins and V. B. White (29), who found stimulation from extracts of corn plants at low concentrations on growth of excised roots, but a toxic effect at higher concentrations. Overbeek, Siu, and Haagen-Smit (23) noted that certain natural extracts were active in *Datura* embryo culture, but that heating, chemical treatments, standing, etc. resulted in loss of activity, which they attributed to a release of toxic substances that inhibited growth of the embryos. Such an explanation might also be applied to the inhibiting or toxic effect of certain of these gall-tissue extracts at the higher concentrations. The toxicity of such products in certain cases may be due to the increased acidity, as was found, for example, with Paris daisy-gall extracts and tobacco tissue (Fig. 2). Since the products from which the extracts were prepared are high in nitrogen, carbohydrate, and ash, certain organic or inorganic compounds may also have been added in such high concentrations as to be toxic to the tissues. Finally, there may be materials in the extracts that counteract otherwise favorable conditions.

In most cases presented here the tissues themselves apparently contained materials that were inhibiting to growth, or toxic in sufficient concentration. The opportunity to investigate the activity of such constituents has been clarified and improved with this tissue culture in a synthetic medium.

These studies suggest the need for a better understanding of the influence on growth of the tissues, for example, of mineral salts, vitamins, and different sources of carbon and nitrogen.

Unpublished work from this laboratory, presented at the St. Louis meetings of the American Association for the Advancement of Science, has shown that various simple organic acids and various amino acids or their salts slow down and even stop the growth of certain tissues *in vitro*. Such compounds seem to deserve more attention as growth regulators or inhibitors.

SUMMARY

The effects of certain crown-gall bacterial metabolites, yeast extract, and crown-gall tissue extracts on the growth *in vitro* of sunflower and tobacco callus tissue have been studied.

Supplements to the basic medium of the fermented, cell-free, bacterial media of virulent and attenuated crown-gall cultures had little influence on the growth of these tissues at low concentrations, but there was a strongly inhibiting effect at the higher concentrations.

At increased concentrations lyophilized virulent and attenuated crown-gall bacterial cells were generally harmful to the growth of both tissues.

The addition of autoclaved marigold crown-gall tissue extract, of unautoclaved tomato-gall extract, and of unautoclaved yeast extract to the basic medium generally stimulated growth at lower concentrations, but inhibited it at higher concentrations. Autoclaved Paris daisy crown-gall tissue extract at all concentrations was beneficial to sunflower tissue, but had either little or an inhibiting effect on tobacco tissue.

REFERENCES

1. Blakeslee, A. F., and Satina, Sophie. New Hybrids from Incompatible Crosses in Datura through Culture of Excised Embryos on Malt Media. Science, **99**:331-334. 1944.
2. Friedman, B. A., and Francis, T., Jr. Gall Formation by *Phytomonas tumefaciens* Extract and Indole-3-acetic Acid in Cultures of Tomato Roots. Phytopath., **32**:762-772. 1942.
3. Gautheret, R. Sur la Possibilité de Réaliser la Culture Indéfinie des Tissus de Tubercules de Carotte. Compt. rend. Acad. d. sc., **208**:118-120. 1939.
4. Geiger, W. B., Jr., and Anderson, R. J. The Chemistry of *Phytomonas tumefaciens*. I. The Lipids of *Phytomonas tumefaciens*. The Composition of the Phosphatide. J. Biol. Chem., **129**:519-529. 1939.
5. Haberlandt, G. Kulturversuche mit isolierten Pflanzenzellen. Sitzungsb. Akad. Wiss. Wien., Math.-Natur. Kl., **111**:69-92. 1902.
6. Henry, B. W., Riker, A. J., and Duggar, B. M. Thiamine in Crown Gall as Measured with the Phycomyces Assay. J. Agr. Res., **67**:89-110. 1943.
7. Hildebrandt, A. C., Riker, A. J., and Duggar, B. M. Effect of Crown-Gall Bacterial Metabolites, Crown-Gall Tissue Extracts, and the Composition of the Medium on Growth *In Vitro* of Excised Tobacco and Sunflower Tissue. Phytopath., **34**:1003-1004. 1944. (Abstract.)
8. Hildebrandt, A. C., Riker, A. J., and Duggar, B. M. Growth *In Vitro* of Excised Tobacco and Sunflower Tissue with Different Temperatures, Hydrogen-Ion Concentrations and Amounts of Sugar. Am. J. Bot., **32**: 357-361. 1945.
9. Just, F. Vergleichende Untersuchungen über die Zusammensetzung von Bierhefe und Futterhefen. Sind Bierhefe und "Kunst"-Hefen gleichwertig? Wochenschrift für Brauerei, **57**:227-231. 1940.
10. Levine, M. Plant Tumors and Their Relation to Cancer. Bot. Rev., **2**:439-455. 1936.
11. Levine, M., and Chargaff, E. The Response of Plants to Chemical Fractions of *Bacterium tumefaciens*. Am. J. Bot., **24**:461-472. 1937.
12. Link, G. K. K., and Eggers, Virginia. Hyperauxiny in Crown Gall of Tomato. Bot. Gaz., **103**:87-106. 1941.
13. Locke, S. B., Riker, A. J., and Duggar, B. M. Growth Substance and the Development of Crown Gall. J. Agr. Res., **57**:21-39. 1938.
14. Locke, S. B., Riker, A. J., and Duggar, B. M. Production of Growth Substance on Peptone Broth by Crown Gall Bacteria and Related Nongall-forming Organisms. J. Agr. Res., **59**:519-525. 1939.
15. Locke, S. B., Riker, A. J., and Duggar, B. M. The Nature of Growth Substance Originating in Crown Gall Tissue. J. Agr. Res., **59**:535-539. 1939.
16. Loo, T. L., and Loo, S. W. Studies on the Culture of Isolated Root Tips under Sterile Conditions. I. The Effect of Leaf Extract on the Growth of Root Tips. Sci. Rep. Nat. Cent. Univ. Ser. B. Biol., **2**:51-79. 1935.
17. Loo, T. L., and Loo, S. W. Studies on the Culture of Isolated Root Tips under Sterile Conditions. II. Further Experiments on the Effect of Leaf Extract on the Growth of Root Tips. Chin. f. Exp. Biol., **1**:189-206. 1936.
18. McIntire, F. C., Riker, A. J., and Peterson, W. H. The Role of Certain Vitamins and Metallic Elements in the Nutrition of the Crown-Gall Organism. J. Bact., **42**: 1-13. 1941.
19. McIntire, F. C., Peterson, W. H., and Riker, A. J. A Polysaccharide Produced by the Crown-Gall Organism. J. Biol. Chem., **143**:491-496. 1942.
20. Nagy, R., Riker, A. J., and Peterson, W. H. Some Physiological Studies of Crown Gall and Contiguous Tissue. J. Agr. Res., **57**:545-555. 1938.
21. Nobécourt, P. Sur la Pérennité et l'Augmentation de Volume des Cultures de Tissus Végétaux. Compt. rend. Soc. d. biol., **130**:1270-1271. 1939.
22. Overbeek, J. van, Conklin, Marie E., and Blakeslee, A. F. Factors in Coconut Milk Essential for Growth and Development of Very Young *Datura* Embryos. Science, **94**:350-351. 1941.
23. Overbeek, J. van, Siu, R., and Haagen-Smit, A. J. Factors Affecting the Growth of *Datura* Embryos *In Vitro*. Am. J. Bot., **31**:219-224. 1944.
24. Riker, A. J. The Relation of Some Chemical and Physico-Chemical Factors to the Initiation of Pathological Plant Growth. Growth, **6**:105-117. 1942.
25. Riker, A. J., and Berge, T. O. Atypical and Pathological Multiplication of Cells Approached through Studies on Crown Gall. Am. J. Cancer, **25**:310-357. 1935.
26. Riker, A. J., Henry, B., and Duggar, B. M. Growth Substance in Crown Gall as Related to Time after Inoculation. J. Agr. Res., **63**:395-405. 1941.

27. Robbins, W. J. Cultivation of Excised Root Tips and Stem Tips under Sterile Conditions. Bot. Gaz., **73**:376-390. 1922.

28. Robbins, W. J., Bartley, Mary, and White, Virginia B. Growth of Fragments of Excised Root Tips. Bot. Gaz., **97**:554-579. 1936.

29. Robbins, W. J., and White, Virginia B. Effect of Extracts from the Corn Plant on Growth of Excised Root Tips. Bot. Gaz., **98**:520-534. 1937.

30. Velick, S. F. The Chemistry of *Phytomonas tumefaciens*. III. Phytomonic Acid, a New Branched Chain Fatty Acid. J. Biol. Chem., **152**:533-538. 1944.

31. White, P. R. Potentially Unlimited Growth of Excised Tomato Root Tips in a Liquid Medium. Plant Physiol., **9**:585-600. 1934.

32. White, P. R. Potentially Unlimited Growth of Excised Plant Callus in an Artificial Nutrient. Am. J. Bot., **26**: 59-64. 1939.

33. White, P. R. A Handbook of Plant Tissue Culture. Lancaster, Pa.: The Jaques Cattell Press. 1943.

34. White, P. R., and Braun, A. C. A Cancerous Neoplasm of Plants. Autonomous Bacteria-Free Crown-Gall Tissue. Cancer Research, **2**:597-617. 1942.

Abstracts

Reports of Research

The Carcinogenicity of *p*-Monomethylaminoazobenzene in Various Diets and the Activity of this Dye Relative to *p*-Dimethylaminoazobenzene. MILLER, E. C., and BAUMANN, C. A. [Coll. of Agric., and McArdle Memorial Lab., Univ. of Wisconsin Med. Sch., Madison, Wis.] *Cancer Research,* **6**:289-295. 1946.

Thirteen groups of 12 to 15 adult Sprague-Dawley rats were fed 0.056% of *p*-monomethylaminoazobenzene or 0.060% of *p*-dimethylaminoazobenzene for 13 to 14 weeks. At this time the livers were examined by laparotomy, and the rats were then continued on the same diets without the dye. The animals were killed for a final tumor count at 22 weeks. Sixty to 87% of rats fed a synthetic diet containing corn oil and *p*-monomethylaminoazobenzene had hepatomas by 22 weeks, while only 30% of the rats fed hydrogenated coconut oil developed tumors by this time. Raising the riboflavin content of the ration from 2 to 10 mgm. per kgm. reduced the tumor incidence slightly; when 20 mgm. per kgm. were fed, only 1 of 14 rats had a hepatoma at 22 weeks. Rats receiving diets which contained 0.3% of guanidoacetic acid, 0.35% of nicotinamide, or 0.3 or 0.5% of choline with *p*-monomethylaminoazobenzene developed approximately the same number of neoplasms as the animals on the control diet, although the methyl acceptors caused a more severe gross cirrhosis than the control diet. The addition of choline minimized the cirrhosis. Analyses of the livers and blood from rats on each of these diets indicated that the methyl acceptors and donor did not alter greatly the levels of *p*-dimethylaminoazobenzene, *p*-monomethylaminoazobenzene, and *p*-aminoazobenzene in the tissues. No liver tumors or gross cirrhosis were found in rats fed 0.106% of *p*-aminoazobenzene with 0.3% of choline for 11 months. When *p*-dimethylaminoazobenzene and *p*-monomethylaminoazobenzene were fed *ad libitum,* the rats receiving the monomethyl compound developed more tumors than those on the dimethyl dye. However, when the two compounds were compared by the paired-feeding technic, the carcinogenic activities were equal.—Authors' abstract.

Increased Incidence of Tumors in Mice After Intravenous Injection of 9:10-Dimethyl-1:2-Benzanthracene. STAMER, S. [Univ. Inst. of Path. Anat., Copenhagen, Denmark] *Acta path. et microbiol. Scandinav.,* **21**:632-639. 1944.

The intravenous injection of an aqueous suspension (1/1000) of 9,10-dimethyl-1,2-benzanthracene once a week for 4 weeks, in a total dose of 2 mgm., was followed by leukemia in 11 and by ovarian cancer in 2 of 51 individuals of the Black mouse strain, while none of their litter-mate controls had neoplastic diseases. This strain is practically "tumor-free," only 1 of a thousand of the animals dying of leukemia. Of 51 Street strain female mice given the injections, 16 died of leukemia, 4 had mammary carcinoma, 5 had cancer of the lung, 3 had "adenoma, suspect of carcinoma" of the lung, and 1 had cancer of the ovary. Among 47 litter-mate controls the incidence of these diseases was 3, 1, 0, 3, and 0, respectively. The injections had no influence on the incidence of mammary cancer or leukemia in female mice of Little's dilute brown (Dlb) strain, or in male and female mice of the Furth strain Aka.—M. H. P.

Further Experimental Studies on Intravenous Injection of 9:10-Dimethyl-1:2-Benzanthracene in Mice (Tolerance, Excretion, Effect on Normal Blood Cells). STAMER, S. [Univ. Inst. for Path. Anat., Copenhagen, Denmark] *Acta path. et microbiol. Scandinav.,* **22**:65-72. 1945.

When a 2.5% aqueous suspension of 9,10-dimethyl-1,2-benzanthracene was injected intravenously in a dose of 4 mgm. on the first day after transplantation of leukemic lymph node suspension into mice of the Aka strain, the development of leukemia was retarded. If the dose was raised to 10 mgm. (2 mgm. on the first, second, third, seventh, and ninth days, respectively), none of the transplants took. The maximum tolerated intravenous dose of this preparation in mice of the Street strain weighing about 20 gm. was 10 mgm. (4 mgm. daily for 2 days, then 2 mgm.); the animals died if 12 mgm. were given (4 mgm. daily for 2 days, then 2 mgm. daily for 2 days). An intravenous injection of 5 mgm. into Street strain mice weighing about 30 gm. caused temporary leukopenia but not anemia; a few mice given 10 to 20 mgm. developed extreme leukopenia and died of enteritis. Excretion studies on male mice of the Dlb strain showed that the time required for excretion of all the hydrocarbon from the body (as indicated by measurement of ultraviolet fluorescence) increased as the size of the intravenous dose increased from 0.1 mgm. to 4.0 mgm. The experiments indicate that while leukemic cells can be killed completely by the hydrocarbon (*e.g.,* in the Aka mice receiving 10 mgm.), the normal white blood cells are more resistant, and may return to the normal number again after complete excretion of the compound, *i.e.,* in about 17 days.—M. H. P.

Deposition of Methylcholanthrene in Some Organs of the Rat. ESMARCH, O. [Aarhus Municipal Hosp., and Radium Centre for Jutland] *Acta path. et microbiol. Scandinav.,* **19**:79-99. 1942.

When 10 mgm. of methylcholanthrene crystals in gly-

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cerol were deposited in the subcutaneous tissue, mammary tissue, striated muscle, peritoneum, spleen, lung, or uterus of rats 6 to 8 weeks old, sarcomas only were produced. Both sarcomas and squamous cell epitheliomas appeared after deposition in the thyroid gland or kidney. Sarcomas, a carcinosarcoma, and a lymphoblastoma were produced by deposition in the liver. Intracerebral injection produced no tumors in the brain but sometimes led to subcutaneous sarcoma formation. The results were not affected by x-ray treatment of the animals before administration of the carcinogen.—M. H. P.

Accelerated Development of Spontaneous Leukemia and Mammary Carcinoma in Mice after Ingestion of Carcinogenic Hydrocarbon. ENGELBRETH-HOLM, J., and POULSEN, O. [Univ. Inst. of Path. Anat., Copenhagen, Denmark] *Acta path. et microbiol. Scandinav.*, **21**: 472-479. 1944.

In mice of the Street strain, the incidence of spontaneous leukemia is 1 to 2% among all the animals, and of mammary tumor, 25 to 30% among the females. The leukemia appears at the age of 10 to 12 months, and the mammary cancer is most frequent in pluriparae that are 14 to 23 months old. One hundred and seventy-five mice (males and females) from this strain were given 9,10-dimethyl-1,2-benzanthracene by stomach tube (2 to 3 mgm. total, in doses of 0.1 mgm. once or twice a week); 142 siblings were used as controls. The mortality was high among the treated animals. At the time the first case of leukosis appeared 68 treated mice were living; 15% of these developed leukosis by the end of 14 months, as compared to 3% in 102 controls. When the first mammary tumor appeared, 22 females were living, and 40% developed tumor by the end of the 14 month observation period, as compared with 7% in the 57 control females. The fundus of the stomach of nearly all the treated animals showed hyperplasia of the squamous epithelium and a tendency to papilloma formation in the mucous membrane; no gastric carcinomas occurred.—M. H. P.

Morphological and Biological Investigations on Benzpyrene Sarcoma in Albino Rats. EKER, R. [Norwegian Radium Hosp.] *Acta path. et microbiol. Scandinav.*, **22**: 1-33. 1945.

A comparative investigation was made of 3 series of benzpyrene tumors in albino rats from a strain that had been inbred for over 10 years. The observations were made up on 106 animals in which the tumor was produced by the subcutaneous injection of 4 mgm. of benzpyrene in 1% olive oil solution, 112 rats given 20 mgm., and 27 rats given 60 mgm. It was found that there was no significant deviation in frequency of the sarcoma types (polymorphous-cell, spindle-cell, mixed-cell, and small spindle-cell). The latent period was shorter for the smallest dosage than for the other two, and was not related to morphological factors or to the tumor growth rate. The tumor growth rate increased slightly with increasing dosage, and decreased within each series as the size of the tumors increased. The frequency of lymph node metastases increased with dosage. Lung metastases, on the whole rare, were somewhat more frequent for the highest dosage than for the other two.

The sarcomas that metastasized generally had a higher atypic value and a lower fiber content than those that did not. The cellular fibrosarcomas had most metastases, the fibrocellular next, and the fibrous forms had none. The frequency of metastases decreased with age of the animals, and was apparently unrelated to the latent period, growth rate, average weight, and duration of the tumors. No relation could be demonstrated between age and morphological factors, latent period, or growth rate.—M. H. P.

The Effects of 9:10-Dimethyl-1:2-Benzanthracene on Transplanted Tumours. STAMER, S. [Danish Anti-Cancer League's Cancer Research Lab., Copenhagen, Denmark] *Acta path. et microbiol. Scandinav.*, **18**:533-557. 1941.

9,10-Dimethyl-1,2-benzanthracene, if given intra-abdominally in toxic doses, inhibited the growth of Crocker sarcoma 180 in Street strain mice, but had no effect on transplanted mammary carcinoma in strain Dlb. In these experiments, the body weights of the control animals were kept about like those of the experimental animals by a reduction in food, in order to eliminate the effect of weight-loss *per se* caused in the experimental animals by the 9,10-dimethyl-1,2-benzanthracene. The literature on the inhibitory effect of carcinogenic hydrocarbons on malignant tumors is reviewed with 33 references.—M. H. P.

The Guinea-Pig as an Experimental Animal in Cancer Research. ESMARCH, O. [Aarhus Municipal Hosp., and Radium Centre for Jutland] *Acta path. et microbiol. Scandinav.*, **19**:100-107. 1942.

Sarcomas developed at the site of injection, within 13 to 21 months, in 3 of 10 guinea pigs that received a subcutaneous injection of 10 mgm. of crystalline methylcholanthrene moistened with glycerol. In 4 of 10 animals given the carcinogen by intraperitoneal deposition, sarcomas developed in the abdominal cavity after 13 to 18.5 months. One of the tumors was successfully transplanted to other guinea pigs, takes occurring in 3 of 10 animals in the first passage and in 7 of 20 in the second passage. The fact that takes were so few in the first passages is attributed to the lack of suitably inbred strains of guinea pigs for such experiments. It would be advantageous if the guinea pig could be used in cancer research, because of its size and immunologic applicabilities.—M. H. P.

The Reaction of Tarred Rabbits to the Myxoma Virus. AHLSTRÖM, C. G. [Path. Inst., Lund, Sweden] *Acta path. et microbiol. Scandinav.*, **17**:394-416. 1940.

In rabbits previously infected with fibroma virus, the intradermal inoculation of myxoma virus usually produces a local skin tumor only, which soon regresses. However, if these animals were given an intramuscular injection of tar before the myxoma virus, the myxoma lesions were larger, continued to grow for some time, and showed a delayed regression in those animals that recovered. The tar effect was seen only if an old, weakened myxoma virus was used. Tar had no influence on the course of myxoma in rabbits not previously infected with fibroma. Tar-treated animals showed no increased susceptibility to myxoma virus, and developed a solid immunity to

fibroma virus as rapidly as did nontarred controls.—M. H. P.

The Influence of "Mucin 1701 W" on Infection with Shope Fibroma and Vaccinia Viruses. CLEMMESEN, J., and ANDERSON, E. K. [State Serum Inst., Copenhagen, Denmark] *Acta path. et microbiol. Scandinav.*, **19**:173-183. 1942.

Granular mucin, prepared by the method of Henderson [*Brit. J. Exper. Path.*, **20**: 1. 1939], when added to the suspending medium of Shope fibroma virus, did not influence the spread of the virus upon intradermal inoculation into young rabbits, but did favor the spread of the virus and the proliferative response to it upon intraperitoneal inoculation, and also increased the local reaction to intradermal injection of either fibroma virus or vaccinia virus. The minimal intradermal dose of either virus necessary for a "take" was reduced by suspending the virus in mucin, or by giving the mucin intravenously.—M. H. P.

Über den Einfluss von Bleitrypanblau auf Teerkrebs bei weissen Mäusen. (Vorläufige Mitteilung). [The Influence of Lead-Trypan Blue on Tar Cancer in White Mice. (Preliminary Communication).] BURSELL, S. [Pharmacol. Inst., Kgl. Univ., Upsala, Sweden] *Acta path. et microbiol. Scandinav.*, **18**:1-19. 1941.

Subcutaneous injection of lead-trypan blue for at least 100 days, starting at the earliest appearance of tar cancers, inhibited metastasis to the regional lymph nodes in male, but not in female mice. The preparation used was that of Woodhouse [*Am. J. Cancer*, **27**: 285. 1936], containing 196.7 mgm. % of lead sulfate (corresponding to 134.4 mgm. % of metallic lead), in which the lead was partially adsorbed on gelatine. Macroscopic examination of the treated animals indicated that the chemical combination with lead destroyed the affinity of trypan blue for tumors.—M. H. P.

Hereditary Tumor-Like Takes in Transplantation of Leukosis in Mice. HOGREFFE, G. [Univ. Inst. of Human Genet., Copenhagen, Denmark] *Acta path. et microbiol. Scandinav.*, **21**:783-800. 1944.

In mice of the Aka strain, which has a very high incidence of spontaneous leukosis, the transplantation of leukotic tissue (lymph nodes, spleen, thymus, liver) from Aka mice with this disease led only to generalized leukosis. No takes occurred in mice of the B strain, which has a low incidence of spontaneous leukosis and which is characterized by pituitary insufficiency as manifested in recessive dwarfism. The animals of the cross Aka × B in the F_2 generation and of strains formed by inbreeding from this generation showed three types of responses: generalized leukosis, tumor formation in the retroperitoneum and mesentery, and generalized leukosis plus tumor formation. The distribution of the types of lesions in the different strains indicates that genetic factors in the hosts determine the outcome of transplantation.—M. H. P.

Mechanical Traumatism and Development of Tumors in Inbred Mouse Strains. ENGELBRETH-HOLM, J. [Univ. Inst. of Path. Anat., Copenhagen, Denmark] *Acta path. et microbiol. Scandinav.*, **21**:775-779. 1944.

Contusion of the lymph nodes in 44 mice of the Aka strain did not increase the incidence of spontaneous leukemia (approximately 60%) in these animals, nor lead to the development of lymphosarcoma at the site of injury. Contusion of the mammary tissue in 49 female mice of the Street strain did not increase the incidence of spontaneous mammary carcinoma (approximately 20%). Testicular tumors, which do not develop spontaneously in either the Aka or Street strain, likewise failed to develop when 46 animals of these strains were subjected to contusion of the testis.—M. H. P.

Induced Resistance in Inbred Homozygous Rats to a Lymphosarcoma Autogenous to the Strain. GOLDFEDER, A. [New York Univ. Coll. of Med. and Bellevue Hosp., and Dept. of Biol., New York Univ., New York, N. Y.] *Proc. Soc. Exper. Biol. & Med.*, **59**:104-109. 1945.

The present study is concerned with the production of resistance in an inbred strain of rats toward a tumor originating in that strain. The tumor employed was a reticulum cell type lymphosarcoma that takes in practically 100% of young rats and rarely regresses spontaneously. Ten to 12 day old tumors were removed under strict aseptic conditions. The conditions for irradiation were: 200 kv., 20 ma., 0.5 mm. Cu, 1.0 mm. Al, and a half-value layer equivalent to 0.9 mm. Cu. Control animals were inoculated with nonirradiated tumor tissue. The effects of irradiation first became manifest with a dose of 2,000 r in air. Although 100% of takes resulted, the normal latent period of 8 to 10 days was extended to 14 days. Increasing the dose to 2,500 r resulted in 60% of takes with a latent period of 18 days, while implants exposed to 3,000 r failed to grow. In 6 experiments, during which x-ray doses varying from 2,200 r to 2,600 r were used, 50 rats were inoculated with the treated tumor tissue. Of 19 negatives, 18 were immune to further transplantation of fresh lymphosarcoma grafts, though in 4 of the 18 tumors appeared but later regressed. The reason only a certain number of the animals became resistant may be that the number of viable tumor cells in each graft varied or that the amount of tumor implant was insufficient to produce a resistant state.—M. B.

On the Occurrence of Diverse Leukotic Conditions in an Inbred Mouse-Strain. KAALUND-JØRGENSEN, O. [Biol. Inst. of Carlsberg Foundation, Copenhagen, Denmark] *Acta path. et microbiol. Scandinav.*, **17**:438-452. 1940.

Four cases of generalized lymphomatosis, 2 of lymphatic leukemia, 1 of myeloid leukemia, 1 of atypical leukemia, and 1 of reticuloma of the spleen were found among 14 mice of 2 generations of an inbred leukemic strain (Ak), received from Furth. The author concludes that in mouse strain Ak the tendency to malignant disease in the hematopoietic system is inherited, while the type of the leukotic changes is determind by nonchromosomal factors.—M. H. P.

Untersuchungen über chronische Lymphadenose bei dänischen Rindern. [Investigations of Chronic Lymphadenosis in Danish Cattle.] EGEHØJ, J. [Skelskør, Denmark] *Acta path. et microbiol. Scandinav.*, **19**:327-378. 1942.

A review of the literature with a 3 page bibliography, and a report of original investigations. Lymphocytomatosis of cattle in Denmark is apparently not due to a

specific virus; a recessive hereditary factor may play a role in its development. The pathology is described in detail.—M. H. P.

Recent Experimental Studies on Leukemia. Furth, J. [Cornell Univ. Med. Coll., New York, N. Y.] *Physiol. Rev.*, **26**:47-76. 1946.

The extensive experimental work done on leukemia during the past few years is summarized in this review. The scope of this work is indicated by the headings under which the material is organized. These include sections on cytology and histology, endocrinology, nutrition, metabolism, and chemical and physical agents. In addition there are discussions of avian leukosis and the virus problem, and also of the somatic mutation hypothesis in the light of recent work in cytogenetics.—R. B.

Relationship of Antibody Content of Normal and Malignant Lymphocytes. Dougherty, T. F., White, A., and Chase, J. H. [Yale Univ., New Haven, Conn.] *Proc. Soc. Exper. Biol. & Med.*, **59**:172-175. 1945.

Antibodies have recently been demonstrated in normal lymphocytes, and the question arose whether they could be found also in the lymphocytes of lymphosarcoma. Male mice, 60 to 80 days old, of the CBA strain (Strong) were used. The lymphosarcoma arose in an estrogen-treated mouse of the C3H strain and is transplantable in 100% of CBA mice. The rapidly growing transplanted tumor kills the host in 2 to 3 weeks but does not metastasize. The antigen used was a filtrate of a 24 hour broth culture of *Staphylococcus aureus*. Each mouse was injected subcutaneously with the toxin solution, either before, or at the time of tumor transplantation. Sera, and extracts of normal and malignant lymphocytes were titrated for their antibody content. Nitrogen analyses of sera and extracts were made by the micro-Kjeldahl method.

Comparison of titers of lymphosarcoma extracts with those of sera and normal lymphocyte extracts indicated that the tumor cells had a somewhat higher antibody content. In cases in which antigen was given only before tumor transplantation, the tumor cells had antibodies; it was concluded that they were capable of securing antibodies from some other source in the body, presumably normal lymphocytes. The growth of an antibody-containing tumor transplant in normal mice was accompanied by the development of new antibody-containing malignant cells. Also normal lymphocytes of hosts receiving such a transplant were shown to contain antibody. There is a reversible exchange of antibody between normal and malignant lymphocytes.—M. B.

On the Nature of Gonadotrophin in Cases of Malignant Tumors of the Testis. Hamburger, C. [State Serum Inst., and Radium Station, Copenhagen, Denmark] *Acta path. et microbiol. Scandinav.*, **18**:457-484. 1941.

Hypophyseal follicle-stimulating gonadotrophin and chorionic gonadotrophin (assayed on rats and mice) were the only gonadotrophins encountered in the urine of more than 150 patients with cancer of the testis. The follicle-stimulating factor was found in about 75% of the cases of seminoma and small-cystic mixed tumor, and the chorionic factor in 3 cases of metastasizing mixed epithelioma of the testis and 1 case of uncertain diagnosis (primary extragenital chorionic epithelioma, or metastases of chorionic epithelioma from a minimal focus in the left testis). One patient with small-cystic mixed tumor (histologically malignant) with embryonal structure and syncytial trophoblast-like cells showed no increase in urinary gonadotrophin excretion shortly after removal of the primary tumor. However, following this, he excreted the follicle-stimulating factor and later, after large metastases had developed in the abdomen, he excreted both types of gonadotrophin.—M. H. P.

Studies on the Excretion of Androgen Substances and Gonadotrophin in Cases of Malignant Tumors of the Testis, Especially Seminoma. Hamburger, C., and Godtfredsen, E. [State Serum Inst., and Radium Station, Copenhagen, Denmark] *Acta path. et microbiol. Scandinav.*, **18**:485-502. 1941.

The urine of 15 of 19 men who had been treated for seminoma of the testis by unilateral castration and x-ray showed contents of hypophyseal follicle-stimulating gonadotrophin that were above normal (*i.e.*, above 50 mouse units per day), and very low contents of androgen (average 10 international units per day, or about $\frac{1}{5}$ the output of normal men). In 12 patients who excreted chorionic gonadotrophin (including those with mixed epithelioma of the testis and chorionepithelioma) the androgen excretion was also low, but was twice as great as in the patients with seminoma.—M. H. P.

Cancerous Changes of a Slow Course and Epithelial Hyperplasia in the Portio Vaginalis of Women, and Similar Changes Produced Experimentally in Guinea-Pigs. Bang. E. Report to Danish Path. Soc., Nov. 24, 1941; from abstr. in *Acta path. et microbiol. Scandinav.*, **19**:315-316. 1942.

Two cases are reported in which changes, "which must be termed superficial cancer," had persisted for more than 2 years before the appearance of manifest cancer in the portio vaginalis. The changes in the portio were characterized by dedifferentiation of the epithelium with abnormal nuclei, sometimes suggesting Bowen's disease, and with ingrowth into gland tubes and connective tissue. In a third case, which remained stationary, the epithelium was likewise dedifferentiated, but the nuclear abnormalities were less pronounced.

By folliculin overdosage and inoculation of infectious substance into the vagina of guinea pigs, the author succeeded in producing metaplasia of the columnar cervical epithelium with ingrowth into the orifices of the gland tubes, corresponding to the changes encountered in simple erosion in women.—M. H. P.

Experimental Tumors after Nerve Section in an Insect. Scharrer, B. [Sch. of Med., West. Reserve Univ., Cleveland, Ohio] *Proc. Soc. Exper. & Med.*, **60**:184-189. 1945; cf. *Science*, **102**:102. 1945; abstr. in *Cancer Research*, **5**:662. 1945.

During a study of the endocrine functions of the corpora cardiaca and allata in the insect *Leucophaea maderae* (Orthoptera), it was found that removal of both corpora allata, together with the posterior portion of the corpora cardiaca, resulted in the development of tumors. The tumors arose mostly in the anterior portion of the alimentary canal and in the salivary reservoir. Rarely, the

salivary glands were also involved. Endocrine disturbances following removal of the corpora allata and cardiaca were suspected as the cause, but further experiments indicated that destruction or interference with some adjacent structure was probably responsible. The recurrent nerve was suspected because of its relationship to the sites of tumor development. Three series of experiments were done. (1) The recurrent nerve was cut behind the corpora cardiaca and allata, leaving the glandular complex intact. (2) The recurrent nerve or its two ventricular branches were cut in the thoracic region. (3) The frontal ganglion, which contains part of the cells of origin of the recurrent nerve, was extirpated. All 3 types of operations resulted in tumors. In series (1) and (2) the incidence was 80% (54 animals used). Hence the neoplasms observed in the anterior portion of the alimentary canal and in the salivary complex were caused by interference with their innervation rather than by a disturbance of the endocrine balance. The tumors consisted of consecutive layers of cells, with nuclei that were pycnotic or irregularly vesicular with little chromatin. Often the cells broke down into a mass of brownish debris. The extent of tissue transformation in the anterior portion of the alimentary canal was such that probably it was incapable of normal function. Animals with these tumors lived from 10 days to several months after operation, with death apparently due to starvation.—M. B.

A Study of Folic Acid Distribution with Respect to Its Possible Relationship to Cancer. Loo, Y. H., and Williams, R. J. [Univ. of Texas, Austin, Tex.] *Univ. of Texas Publication No. 4507:* 123-134. 1945.

Assays of folic acid in the tissues of rats bearing Walker carcinoma 256 transplants and normal controls indicated that the acid is bound in various types of linkages. One type, present in spleen, skeletal muscle, and tumor tissues, is hydrolyzed by clarase; another, found in the liver of normal and cancer-bearing rats, is hydrolyzed by liver enzymes. A third, found in normal liver, is hydrolyzed by a combination of clarase and liver enzymes in the presence of phosphate buffer at pH 7 and 4% NaCl, but not in the presence of acetate buffer at pH 4.5. The linkage of folic acid to liver tissue differed strikingly in cancer-bearing rats to that in the controls. Clarase digestion of normal liver tissue released much more folic acid when the digestion was carried out in a phosphate-NaCl buffer than when it was carried out in acetate medium, while this was not true for liver tissue from cancer-bearing rats. The concentration of folic acid in a muscle adjacent to an implanted tumor in one hind limb was as high as that in the muscle of the normal hind limb, indicating that the growing tumor does not deplete the surrounding tissue of this material.—M. H. P.

Serological Analysis of a High-Molecular Crystallizable Protein in Myeloma Serum. Packalén, T. [Sero-Bacteriol. Inst., Helsingfors Univ., Helsingfors, Finland] *Acta path. et microbiol. Scandinav.,* **17**:263-272. 1940.

A serum protein of high molecular weight, crystallizing spontaneously from the blood serum of a patient with myeloma, differed from the proteins of normal serum in precipitation reactions against rabbit antisera. In anaphylaxis experiments performed in guinea pigs, however, this serological specificity did not appear distinct.—M. H. P.

Fibromatous Skin Lesions Produced by Repeated Blood Serum Injections in the Human. Marshall, W. [Spring Hill Coll., Mobile, Ala.] *Am. J. Surg.,* **69**:338-343. 1945.

Using himself as a subject, the author demonstrated the production of fibromatous skin lesions at the sites of repeated intracutaneous serum injections totalling 14 and 17 injections in two series. The sera used were: (1) that from a "Wassermann-fast" patient and, (2) his own blood serum; both were preserved with ½% phenol. Control injections of saline with ½% phenol were given into the opposite thigh. At the end of the injection periods (17 and 9 days respectively) biopsies of the experimental areas showed a condensation and thickening of the collagen material in the subepithelial tissue. The control areas were normal. The author postulates a positive chemotropism in the production of fibromatous growth, the presence of blood serum extravasations having the ability to attract fibroblasts.—W. A. B.

On the Metastasis Problem. Ostenfeld, J. [Biol. Inst. of Carlsberg Foundation, Copenhagen, Denmark] *Acta path. et microbiol. Scandinav.,* **19**:209-219. 1942.

According to the observations of Blumenthal and others, transplantable tumors can be transmitted by cell suspensions of organs apparently free from metastasis. The author produced tumors in mice by the subcutaneous injection of minced lung, spleen, liver, kidney or blood from mice bearing either the Ehrlich carcinoma or other tumors. These results are attributed to the transmission of tumor cells (escaping observation on microscopic study) rather than to a virus, since exposure of mice bearing the Ehrlich carcinoma to x-rays in a dose of 6,000 r (a dose believed to kill tumor cells but not viruses) rendered their lung tissue no longer capable of causing tumor on subcutaneous injection into other mice.—M. H. P.

Clinical and Pathological Reports

Clinical investigations are sometimes included under Reports of Research

Heredity

Familial Occurrence of Leukemia. Hogreffe, G. [Univ. Inst. for Human Genet., Copenhagen, Denmark] *Acta path. et microbiol. Scandinav.*, **22**:89-101. 1945.

A family is described in which 2 sisters died of myelogeous leukemia and their nephew of plasmocytic reticulosarcoma. The literature on familial occurrence of leukemia is reviewed, with bibliography and tables.—M. H. P.

Multiple Tumors

Multiple Primary Malignancy. Goldman, C. [Brooklyn Cancer Inst., Brooklyn, N. Y.] *Am. J. Surg.*, **69**:265-269. 1945.

The case reported is that of a woman dying at 60 years of age who had had a mastectomy at the age of 41 for a medullary carcinoma of the breast. At the age of 58 she had undergone an operation with removal of an ulcer, which had developed in the scar of the previous incision following irradiation. The microscopic diagnosis on this second tumor was squamous cell carcinoma. She later developed a transitional cell carcinoma of the cervix, for which she received x-ray therapy at the Memorial Hospital. One year later, she was found to have a papillary adenocarcinoma of the rectum. Removal was not attempted, and she died shortly after the fourth carcinoma was diagnosed.—W. A. B.

Diagnosis

Autopsic Evaluation of the Clinical Diagnosis "Cancer of the Stomach," with Special Reference to Its Value to the Cancer Statistics. Hansen, J. L. [Kommune Hosp., Copenhagen, Denmark] *Acta path. et microbiol. Scandinav.*, **21**:731-746. 1944.

Among 8,633 autopsy records accompanied by clinical diagnoses, relating to adult patients who died in the surgical and medical departments of a Danish hospital during 1915 through 1935, there were 396 in whom a clinical diagnosis of cancer of the stomach was supported, 132 in whom this clinical diagnosis was found to be erroneous, and 200 in whom cancer of the stomach was revealed after having been missed clinically. In the group of 200 patients last mentioned, 71 had not manifested symptoms of the disease, and 129 had shown symptoms that were erroneously interpreted. If the symptom-free cases are omitted from consideration, the false positive clinical diagnoses (132) are found to be almost exactly equal numerically to the false negative ones (129). Hence (with correction for symptom-free cases) clinical diagnoses alone can be expected to give a fairly reliable index to the frequency of fatal gastric cancer in the population.—M. H. P.

Skin and Subcutaneous Tissues

The Paget Cell; Its Structure, Occurrence and Significance. Meirowsky, E., and Keys, S. *Proc. Roy. Soc. Med.*, **38**:495-499. 1945.

The author expresses his views concerning the nature of the Paget cell. He concludes that "the process occurring in the Paget cell is a normal response of the epithelial cell towards different kinds of animate and inanimate stimuli among which are tar, x-rays, sunlight, viruses, and the unknown causes of cancerous conditions, psoriasis, and lichen planus."—L. W. P.

The Principle of Excision and Dissection in Continuity for Primary and Metastatic Melanoma of the Skin. Pack, G. T., Scharnagel, I., and Morfit, M. [Memorial Hosp., New York, N. Y.] *Surgery*, **17**:849-866. 1945.

The scope of the operation for removal of a melanoma of the skin should be planned to enable the surgeon to remove the primary lesion and the regional lymph nodes in one encompassing excision of skin and deeper structures. When the lesion is on the trunk, this may be accomplished without grafting, but grafting is necessary if the lesion is situated on the hands, feet, lower legs, forearms, head, or neck. When regional nodes are removed in this manner, they have been found to contain microscopically identifiable melanoma even when there was no clinical evidence of metastases; this was the case in 2 of 7 instances of elective groin dissection for melanomas of the extremities or genitalia.

Six cases are reported here.—W. A. B.

Glomus Tumor (Glomangioma, Angioneuromyoma, Glomal Tumor). Pohl, J. F. [Minneapolis, Minn.] *Journal-Lancet*, **65**:253-254. 1945.

Case report. The tumor was in the left fourth finger, and involved the bone.—M. E. H.

Glomangioma. Beatty, W. M. [Liverpool Roy. Infirmary, Liverpool, England] *Lancet*, **249**:137-138. 1945.

Three typical examples of the glomus cell tumor are described in patients aged 25, 54, and 39 respectively, and an account of the normal neuromyoarterial glomus is given. A plea is made for the more general recognition of this small tumor, which presents itself clinically as a bluish, soft, subcutaneous mass with the characteristic symptom of lancinating paroxysmal pain that is relieved with uniform success by simple excision of the growth under local anesthesia. Malignant change is unknown. —L. W. P.

Hemangioendothelioma of the Skin. Caro, M. R., and Stubenrauch, C. H., Jr. [Univ. of Illinois Coll. of Med., Chicago, Ill.] *Arch. Dermat. & Syph.*, **51**:295-304. 1945.

Report of a case in which the tumor appeared on the scalp at the site of trauma, spread slowly over the face, scalp, and neck, and produced late metastases in the cervical lymph nodes.—J. G. K.

Mixed Tumors of the Skin. Report of Cases, with a Consideration of the Histogenesis of Mixed Tumors of Organs Derived from the Ectoderm. Morehead, R. P. [Bowman Gray Sch. of Med. of Wake Forest Coll., and Baptist Hosp., Winston-Salem, N. C.] *Arch. Path.*, **40**:107-113. 1945.

Observations made on 4 cases suggest to the author that mixed tumors arising in the skin, the breast, and the salivary glands are histogenetically similar.—J. G. K.

Disseminated Visceral Idiopathic Hemorrhagic Sarcoma (Kaposi's Disease): Report of Case with Necropsy Findings. Nesbitt, S., Mark, P. F., and Zimmerman, H. M. [Yale Univ. Sch. of Med., New Haven, Conn.] *Ann. Int. Med.*, **22**:601-605. 1945.

This case is of particular interest because of the involvement of the thyroid gland and brain, and the presence of disseminated visceral lesions in the absence of dermal manifestations.—J. G. K.

Breast

Some Unusual Aspects of Cancer of the Breast. Daland, E. M. [Pondville Hosp., Wrentham, and Tumor Clin. of Massachusetts Gen. Hosp., Boston, Mass.] *New England J. Med.*, **233**:515-519. 1945.

Examples are given of the importance of investigating vague symptoms in the breast, even in young girls. The necessity of radical mastectomy is emphasized, but several case reports of simple mastectomy and the indications for it are given. A case of recurrence 34½ years after radical mastectomy is reported. Two cases of persistent recurrence of low grade cancer over periods of 24 and 10 years, respectively; are reported in detail.—C. W.

Carcinoma of the Breast: A Study of 37 Cases. Kent, G. B., and Sawyer, K. C. [Denver, Colo.] *Rocky Mountain M. J.*, **42**:672-676. 1945.

Among the 37 cases of carcinoma of the breast followed by the authors, 90.9% were classed as grade 3 or 4 or highly malignant. The 3 year survival rate was 75%; 5 year was 50%, and 15% of patients have survived 10 years. The results from prophylactic roentgen therapy are not impressive. Pain was a more common complaint in these cases than previously supposed to occur in carcinoma of the breast.—M. E. H.

Giant-Cell Tumours of the Breast. Engelbreth-Holm, J. [Cancer Research Lab. of Danish Anti-Cancer League, Copenhagen, Denmark] *Acta path. et microbiol. Scandinav.*, **17**:506-523. 1940.

A report of 2 cases, and a review of 5 from the literature.—M. H. P.

Eye

Orbital Tumours. Jackson, H. *Proc. Roy. Soc. Med.*, **38**:587-594. 1945.

The differential diagnosis is intimately related to that of "unilateral proptosis," the lesions most likely to be confounded being exophthalmic ophthalmoplegia, certain generalized bone diseases, meningocele or encephalocele, and vascular lesions of aneurysmal or of arteriovenous fistulous nature. Proptosis, either unilateral or bilateral, has been found in association with subdural hematoma, basal tumors of the anterior and middle fossae, tumors of the cerebello-pontine angle, ventricular tumors, and occipital tumors. An intracranial lesion that encroaches upon but does not extend into the orbit, such as a cyst of the jaw, may produce proptosis owing to deposition of bone on the surface coincident with its absorption adjacent to the lesion. The so-called "pseudo-tumor of the orbit" is an infective granuloma. Apart from the outstanding unilateral proptosis, other symptoms discussed include visual deterioration, pain, tumor formation, ptosis, edema of the eyelids, chemosis, cranial nerve palsies, an audible bruit, and a discharging sinus. Reference is also made to the ophthalmoscopic examination, the duration of the disease, the pathology of the various lesions found and to the radiographic appearances, laboratory investigations, and treatment.—L. W. P.

Orbital Tumours. Meadows, S. P., et al. *Proc. Roy. Soc. Med.*, **38**:594-600. 1945.

A general discussion, with case reports, on a variety of orbital tumors, some with intracranial extension.—L. W. P.

Glioma of the Optic Nerve. Katzin, H. M. [Mt. Sinai Hosp., New York, N. Y.] *J. Mt. Sinai Hosp.*, **11**:332-335. 1945.

A case is reported of glioma of the optic nerve, with operative exploration, biopsy, and treatment. The progressive nature of this neoplasm is stressed, and its frequent association with von Recklinghausen's disease noted. The apparent benefit from radiation, seen in this case at present, may be temporary. If it proves to be lasting, the observation is a significant one. Most cases are found to be inoperable.—Author's summary. (A. Cnl.)

Therapy—General

Surgical Treatment of Cancer of the Larynx. Jackson, C. L., and Norris, C. M. [Philadelphia, Pa.] *Laryngoscope*, **55**:196-215. 1945.

The author briefly mentions the various types of surgical procedures, methods of irradiation, and their combination employed in the treatment of cancer of the larynx. He enumerates certain indications for surgical treatment. Irradiation is preferred for inoperable growths and for lesions unsuitable for laryngofissure where laryngectomy is contraindicated because of the patient's condition. The technic of laryngofissure and laryngectomy as practiced at Temple University Clinic is described in detail. The postoperative care and complications are discussed. Of a total of 148 laryngofissures performed over a period of 14 years, not a single patient has died, nor has there been a single operative fatality in the last 70 laryngectomies. Of 150 patients with cancer of the larynx treated by all methods between 1930-1937 a "5 year cure" rate of 64% was obtained. Surgical treatment for intrinsic lesions was given 101 of these individuals; 75% were "cured." Laryngofissure resulted in a "5 year cure" rate of 80%, while 69% of intrinsic lesions subjected to laryngectomy were "cured."

The bibliography includes 25 papers. There are 5 figures illustrating the technic of laryngectomy.—A. M.